DECLARATION OF ORIGINALITY

I declare that the work presented herein and the composition of this thesis is my own.

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HIGH MOLECULAR WEIGHT FORMS OF ALKALINE PHOSPHATASE

By

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ABSTRACT

Two high molecular weight complexes of alkaline phosphatase in serum have been studied with a view to discovering their nature and evaluating their respective roles in clinical diagnosis. One of these ('slow band alkaline phosphatase') was shown by inhibition and immunochemical studies to consist of liver or bone alkaline phosphatase attached to immunoglobulin G. It appears only rarely in serum and is not useful in clinical diagnosis. The other complex ('high molecular weight alkaline phosphatase') was more intensively investigated. Following purification from serum and bile, kinetic studies showed that it resembled the normal liver isoenzyme in serum and the low molecular weight isoenzymes in bile. Other enzymes which derive from the liver cell membrane were found to have similar high molecular weight complexes which had similar electrophoretic and chromatographic properties. All the complexes were destroyed by butanol extraction and converted to lower molecular weight forms by detergents. Immunochemical experiments showed that all the complexes shared antigenic determinants with lipoprotein X, although in many sera large amounts of high molecular weight alkaline phosphatase were present in the absence of detectable lipoprotein X. Using

the results of these experiments and other research in a variety of fields, it is suggested that high molecular weight alkaline phosphatase may consist of a family of membrane fragments of two main interconvertible forms: one equivalent to biliary lipoprotein, the other equivalent to lipoprotein X. Only the former is present in bile, but either of the two may be present in serum depending on various conditions. Clinical evaluation showed that measurement of the activity of high molecular weight alkaline phosphatase in serum was a sensitive test for obstructive liver disease and was particularly useful in diagnosing liver metastases.

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CHAPTER 1

INTRODUCTION

Alkaline phosphatase (E.C.3.1.3.1.), first described by Robison (1923), is a dimeric zinc-containing glycoprotein which catalyses the hydrolysis of a wide range of phosphomonoesters at alkaline pH. The amino acid sequence of the enzyme has not yet been established but the active site probably contains a serine residue to which the phosphate group of the substrate binds during catalysis to form a phosphoryl-enzyme intermediate (Engstrom, 1961). Magnesium ions are necessary for this function.

Most human tissues contain alkaline phosphatase (ALP), intestine, liver, kidney, bone and spleen being particularly rich sources. The enzymes derived from these tissues share the same broad catalytic properties but differ in their detailed kinetic and physical characteristics. Historically, the term 'isozyme' or 'isoenzyme' has been used to denote the different molecular forms in which proteins may exist with similar enzymatic specificity (Markert and Møller, 1959) and this term has been applied to the ALPs derived from different tissues in a single species e.g. liver isoenzyme, bone isoenzyme etc. However, recent recommenda-

tions (the International Union of Pure and Applied Chemistry and the International Union of Biochemistry Commission on Biochemical Nomenclature, 1976) reserve the term 'isoenzyme' for those multiple forms of enzymes arising from genetically determined differences in primary structure and not those derived by modification of the same primary sequence. Such information is not yet available for ALP. Moreover, the Commission discourages the application of labels on the basis of tissue distribution since confusion may arise on account of species variation. Instead, they recommend labelling on the basis of electrophoretic mobility. Nevertheless, the terms liver isoenzyme, bone isoenzyme etc. will be employed for the ALP forms deriving from these tissues for the following reasons:

1) only the human species is considered in this thesis
2) the relative mobilities of the ALP molecular forms vary according to the electrophoretic medium employed
3) it seems desirable to maintain continuity with the literature and established practice.

In this context, the term 'liver isoenzyme' denotes the ALP of relatively low molecular weight (mol wt), namely approximately 200 000, which derives from the liver.

1.1. THE ISOENZYMES OF ALKALINE PHOSPHATASE

1.1.1. Function of isoenzymes in tissues

The function of the various ALP isoenzymes is largely uncertain. The bone isoenzyme appears to play a role in the calcification and resorption of bone by a combination of local supersaturation of phosphate following hydrolysis of organic phosphate esters, and removal of the inhibitor pyrophosphate (Russell et al, 1969). The intestinal isoenzyme is probably involved in calcium and fat absorption (Warnock, 1968; Norman et al, 1970). The localisation of the enzyme on the small intestinal mucosa, the proximal convoluted tubule of the kidney, the syncytiotrophoblast of the placenta and the sinusoidal and bile canalicular membranes of the hepatocyte all suggests a transport function. Pekarthy et al (1972) have proposed that liver ALP is involved in the transhepatic transport of choline by hydrolysis of phosphorylcholine.

1.1.2. Serum isoenzymes

There are three ALP isoenzymes which appear in the serum of healthy individuals: liver, bone and intestinal. The placental isoenzyme appears in the serum of women in the last trimester of pregnancy. These isoenzymes have similar mol wts, of the order of 200 000, but they can be

distinguished using a number of criteria:

1) Electrophoretic mobility. The liver, bone and intestinal isoenzymes migrate with different mobilities towards the anode during electrophoresis (see section 1.3.1.). If the serum is pre-incubated with neuraminidase, the mobility of the liver and bone isoenzymes is greatly decreased, indicating loss of sialic acid residues. The intestinal isoenzyme, on the other hand, does not alter its mobility, indicating either that it has no sialic acid residues or that they are inaccessible (Robinson and Pierce, 1964).

2) Inhibition. The intestinal and placental isoenzymes are strongly inhibited by L-phenylalanine whereas the liver and bone isoenzymes are little affected (Fishman et al, 1963). On the other hand, the liver and bone isoenzymes are strongly inhibited by L-homoarginine whereas the intestinal and placental isoenzymes are little affected (Lin and Fishman, 1972). Both these inhibitions are of the uncommon uncompetitive type in which the inhibitor combines only with the enzyme-substrate intermediate.

3) Inactivation by urea and heat. Liver ALP can be distinguished from bone ALP and from the intestinal and placental isoenzymes by their differential stability to

urea and heat. The isoenzymes are inactivated by urea, which ruptures hydrogen bonds, in the following order of lability: bone > liver > intestinal > placental (Bahr and Wilkinson, 1967). In fact, the liver isoenzyme is slightly activated at low urea concentrations, unlike the other isoenzymes. Heating at 56°C inactivates the isoenzymes in the following order of lability: bone > liver > intestinal > placental (Petit Clerc, 1976). At 65°C, all the isoenzymes except the placental are rapidly inactivated.

4) Immunological properties. Different results have been obtained depending upon the purity of the isoenzymes used to raise antisera. Liver and bone ALP may belong either to a single immunological class (Boyer, 1963) or to two separate classes (Sussman et al, 1968), while the intestinal and placental isoenzymes form two further distinct classes (Boyer, 1963; Sussman et al, 1968).

1.2. THEORIES FOR SERUM ALKALINE PHOSPHATASE ELEVATIONS IN DISEASE

Total serum ALP activities are increased in a number of diseases affecting bone (e.g. Paget's disease, osteomalacia) and liver (e.g. extrahepatic obstruction, metastatic liver disease). Historically, there have been a number of theories to account for this increase:

1) The retention theory, proposed by Gutman (1959), which

postulated that all the ALP in serum derived from bone, and that the principal method of removal of the enzyme from the circulation was by excretion via the liver into bile. The increase seen in osteoblastic bone disease was a consequence of an increased rate of release of this ALP into the circulation whereas the increase seen in liver disease, particularly of a cholestatic type, was held to be a consequence of failure of the liver to excrete this bone enzyme into bile.

2) The hepatogenic theory (Hill and Sammons, 1967) which suggested that the increase in ALP in liver disease arose, not from failure to excrete a bone enzyme, but from release of an enzyme deriving from the liver into the circulation, possibly because it could not pass down the biliary tract and was therefore regurgitated into serum via canicular-sinusoidal connections. It was suggested that both the bone enzyme and the liver enzyme were removed from the circulation by catabolism rather than by excretion, in a similar way to other plasma proteins.

3) The induction theory, first proposed by Rolin et al (1962), which was a refinement of the hepatogenic theory. This suggested that the mechanism of the increased rate of release of liver ALP into plasma during cholestasis was by induction of synthesis rather than either release

of pre-existing liver ALP into the circulation or activation of the enzyme in the liver and circulation.

The retention theory has now been abandoned for a number of reasons:

a) The demonstration of different isoenzymes in serum, the properties of which closely resembled those of isoenzymes extracted from liver, bone and intestine (Hodson et al, 1962), made it clear that not all ALP derives from bone. In bone disease the bone isoenzyme in serum may be elevated whereas in liver disease the liver isoenzyme may be raised.

b) Injection of plasma from dogs with a ligated common bile duct into normal recipient dogs resulted in continued high activities in the recipient dogs for 48 hours with very little of the infused ALP being excreted in bile (Cantarow and Miller, 1948). Similarly injection of purified human placental ALP (measured by its heat stability) into humans demonstrated that in both normal subjects and patients with biliary obstruction with raised endogenous ALP serum levels, the placental isoenzyme followed a decay curve in serum similar to that observed for other plasma proteins (Clubb et al, 1965).

c) Little ALP activity is present in normal hepatic bile,

insufficient to identify bile as a major route of excretion. Furthermore, the isoenzyme pattern of bile differs from that of normal serum.

The accumulated evidence therefore led to the adoption of the hepatogenic theory.

Evidence for the induction theory stems from bile duct ligation experiments in animals. Polin et al (1962) first demonstrated that ligation of one hepatic duct in dogs caused a) an increase in ALP in bile collected from the other duct, b) a 10-fold increase in liver ALP, c) an increase in serum ALP in the absence of hyperbilirubinaemia. They suggested that induction rather than decreased excretion caused these elevations. Similar conclusions were drawn following experimental biliary occlusion in isolated perfused cat livers (Sebesta et al, 1964). Further experiments involving bile duct ligation in rats demonstrated that the increase in serum ALP lagged behind the increase in liver ALP and both increases could be prevented <u>in vivo</u> using inhibitors of protein synthesis such as ethionine, cycloheximide and actinomycin D (Börnig et al, 1967; Kaplan and Righetti, 1969; Kryszewski et al, 1973). The induction theory is therefore now widely accepted. It is presumed that the inducer is some chemical substance, normally excreted in bile, which rises

in concentration during biliary obstruction. The identity of this substance has not been proved although Pekarthy et al (1972) suggest phosphorylcholine as a possibility.

1.3. ISOENZYME PATTERNS IN HEALTH AND DISEASE

1.3.1. Electrophoretic techniques

Electrophoretic media which have been employed to separate ALP isoenzymes can be divided into two classes: a) those which do so principally on the basis of charge differences and b) those which do so on the basis of a combination of charge differences and molecular sieving effects. Into the first category fall paper (Hill and Sammons, 1967), agar, agarose (in the concentrations generally employed, Demetriou and Beattie, 1971) and cellulose acetate (Fritsche and Adams-Park, 1972). Starch gel (Chiandussi et al, 1962; Hodson et al, 1962), polyacrylamide gel (Smith et al, 1968) and thin-layer Sephadex G200 (Inglis et al, 1968) fall into the second group.

In Health. In normal healthy individuals, only a limited number of isoenzyme bands can be visualised in any of these media. In general, the liver isoenzyme has the fastest mobility. It may or may not be resolved from the bone isoenzyme (only polyacrylamide gel can completely

resolve them) which tends to be a broader more diffuse band running immediately behind the liver isoenzyme. In some individuals, particularly in those of O or B blood groups who are also secretors, the intestinal isoenzyme is also present, running with lower mobility than either the liver or bone isoenzymes. In starch gel alone, a further band may be seen in healthy individuals, migrating in the βlipoprotein position. This band may be intensified in either liver or bone disease (Chiandussi et al, 1962; Hodson et al, 1962; du Buisson and Pepler, 1966; Hill and Sammons, 1967).

In disease, various characteristic isoenzyme patterns may be seen.

Cancer-specific isoenzymes. Carcinoma of a wide variety of primary sites may give rise to a new band corresponding to the Regan isoenzyme. This is a cancer-specific enzyme which has properties identical to those of the placental isoenzyme, including inhibition by L-phenylalanine, stability to heat and immunological properties (Fishman, Inglis, Stolbach and Krant, 1968).

An isoenzyme similar to the Regan isoenzyme has been described in patients with carcinoma of the liver or gastrointestinal tract only. This is the Regan variant or Kasahara isoenzyme (Higashino et al, 1972; Crofton

and Smith, 1978). It is much rarer than the Regan isoenzyme and differs from it in its lower heat stability and higher electrophoretic mobility.

Bone disease. Bone disease of an osteoblastic type may be accompanied by an increase in the bone isoenzyme band in all the electrophoretic media.

Liver disease. The liver isoenzyme may be increased, particularly in liver disease of an obstructive type. In addition, a new band may appear on electrophoresis in various media (Table 1.1). Any evidence for the identity or non-identity of these bands has been very limited and purely circumstantial e.g. Fritsche and Adams-Park (1972) found that the same sera which possessed an isoenzyme with pre-liver mobility on cellulose acetate also had an origin band in polyacrylamide gel. No systematic studies have been attempted.

Bile. Bile has been less investigated than serum. Electrophoresis in paper gives an origin band only (Hill and Sammons, 1967). In cellulose acetate, bile has two isoenzyme bands, one in the bone isoenzyme position and one with a pre-liver isoenzyme mobility (Fritsche and Adams-Park, 1972). In starch gel, various combinations of bands have been observed, frequently but not invariably similar to the patterns seen in serum from patients with liver

TABLE 1.1.

Position of extra electrophoretic bands which may appear in cholestatic liver disease

Electrophoretic medium	Position of extra band	References
Cellulose acetate	Pre-liver ALP	Fritsche and Adams-Park (1972)
Agarose: 1%	Immediately behind liver ALP in bone ALP position	Demetriou and Beattie (1971)
2.4%	Between intestinal ALP and origin	Ewen (1974)
Paper	Origin	Hill and Sammons (1967)
Thin-layer Sephadex G200	Between liver ALP and origin	Inglis et al. (1968)
Polyacrylamide gel (5%)	Origin	Walker and Pollard (1971)
Starch gel	Origin	Chiandussi et al (1962); Hodson et al (1962); du Buisson and Pepler (1966); Pope and Cooperband (1966); Hill and Sammons (1967).

disease (Chiandussi et al, 1962; Pope and Cooperband, 1966; Hill and Sammons, 1967).

1.3.2. Gel filtration chromatography

Estborn (1964) first applied gel filtration chromatography to normal serum ALP: two peaks were obtained on Sephadex G200, the major peak eluting with proteins of approximately 200 000 mol wt and a minor peak with proteins of mol wt greater than 800 000 (i.e. with the α_2 and β_2 macroglobulins, and the α and β lipoproteins) in the void volume. Akedo et al (1967) showed that the void volume peak showed an increase relative to the lower mol wt peak in liver disease but gave no quantitative analysis of the results. Later, Fennelly et al (1969) measured the void volume peak and found that it comprised 2% of the total activity in controls, 1% in non-metastatic bone disease, 6% in metastatic bone disease, 16% in non-metastatic liver disease and 36% in metastatic liver disease. This was therefore a high mol wt ALP which seemed to be virtually specific for liver disease. In bile, nearly all the ALP activity was in the void volume peak (Price and Sammons, 1974).

Although the appearance in liver disease of the new electrophoretic bands summarised in Table 1.1 and the Sephadex G200 high mol wt peak provided circumstantial

evidence that they might be related, no studies have been performed to relate electrophoretic to gel filtration findings except one which showed that the excluded fraction on Sephadex G200 remained at the origin on polyacrylamide gel electrophoresis (Price and Sammons, 1974).

Similar void volume peaks on Sepharose 4B chromatography have been described for membrane marker enzymes adenosinetriphosphatase, leucine aminopeptidase and 5'nucleotidase by Shinkai and Akedo (1972). These workers suggest that high mol wt ALP consists of a multienzyme complex which represents a fragment of plasma membrane released from the liver in liver disease. This hypothesis is tested and explored in this thesis.

1.4. A RARE HIGH MOLECULAR WEIGHT FORM OF ALKALINE PHOSPHATASE

Using starch gel electrophoresis, an atypical slowly migrating zone of ALP activity (slow band) was first reported by Streifler et al (1972) in a patient with ulcerative colitis. This was followed by a paper by Qirbi and Moss (1975) which described the occurrence of a similar slow band on polyacrylamide gel electrophoresis in a further 3 out of a total of 54 patients with ulcerative colitis or Crohn's disease. In each case the slow band formed between 50 and 100% of the total ALP activity. Isolated reports of similar slow bands have also appeared

(Nagamine and Ohkuma, 1975; Lee, 1976). Dingjan et al (1975) found a slow band in 7 out of 2500 sera submitted for routine ALP analysis.

Preliminary investigations in a series of patients with the slow band revealed that it had a mol wt intermediate between that of the liver isoenzyme and the high mol wt ALP considered elsewhere in this thesis. It has therefore been included in the thesis as a separate chapter (Chapter 6). To avoid confusion with the high mol wt ALP which forms the principal object of study of the thesis, it has been termed 'slow band ALP'. The aims of the investigations into this slow band ALP were:

1) to assess its clinical significance
2) to compare its properties with those of the liver, bone and intestinal isoenzymes in order to shed light on its nature
3) to discover whether it was a simple aggregate of lower mol wt forms or whether it was a complex of ALP with some other protein, carbohydrate or lipid.

1.5. OTHER SERUM ENZYMES SHOWING INCREASED ACTIVITY IN CHOLESTATIC LIVER DISEASE

Besides ALP, other enzymes may show increased activity in the serum of patients with intra- or extrahepatic obstruction. Those which have been most commonly

measured for diagnostic purposes include γ glutamyltransferase (γGT), leucine aminopeptidase (LAP) and 5'nucleotidase (5'NT). All three are membrane-marker enzymes.

1.5.1. γ Glutamyltransferase (E.C.2.3.2.2.)

γGT is present in a large number of tissues, but chiefly in the kidney, pancreas, liver and prostate. Within the liver, it is principally located on the bile canalicular membrane of the hepatocyte, but some may also be found within the periportal hepatocytes (Rosalki, 1975). γGT is also to be found in bile. The reaction which it catalyses is:

$$(\gamma\text{-L-glutamyl}) - \underset{\text{amino acid}}{\text{peptide}} + \rightleftharpoons \text{peptide} + \underset{\text{amino acid}}{\gamma\text{-L-glutamyl} -}$$

It is thought that it may play a role in the transport of amino acids and also in facilitating the transfer of amino acids from t-RNA to ribosomal protein.

γGT is a sensitive enzyme for the detection of liver disease but it is not helpful for distinguishing between different types of liver disease. It is particularly useful in detecting cholestasis and cirrhosis, especially when the latter is of the alcoholic type. There is a good deal of evidence to suggest that alcohol induces synthesis of the enzyme, although this is not yet proven. Phenobarbitone also appears to induce γGT synthesis together with synthesis of other microsomal enzymes

(Rosalki, 1975). However, bile duct ligation experiments in rats have shown that the rise in serum γGT precedes the rise in liver γGT which occurs considerably later than the rise in liver ALP. The effect of inhibitors of protein synthesis on this late rise in liver γGT could not be assessed since the rats could not be maintained for long enough on these toxic compounds (Kryszewski et al, 1973).

Isoenzyme analysis of γGT has frequently been undertaken in a number of electrophoretic media in an attempt to make the assay more specific for different types of liver disease. Depending on the medium used, a number of bands may be observed in normal sera while in liver diseases of various types a complex variety of additional bands may appear (Rutenberg et al, 1967; Patel and O'Gorman, 1973; Rosalki, 1975). No comparisons with the ALP isoenzyme bands have been undertaken, but γGT activity has been observed at the origin during starch gel and polyacrylamide electrophoresis (c.f. Table 1.1), in addition to many other bands of activity (Orlewski and Szczeklik, 1967; Azzopardi and Jayle, 1973). In general, isoenzyme analysis of γGT has not been found to be useful in clinical diagnosis.

Sephadex G200 gel filtration of serum generally shows 3 peaks of γGT activity, the first coinciding with the

void volume (Jacyszyn and Laursen, 1968). This high mol wt peak may give bands either at the origin or in the βlipoprotein position on starch gel electrophoresis, and is increased in obstructive jaundice (Orlewski and Szczeklik, 1967; Rosalki, 1975).

1.5.2. Leucine aminopeptidase (E.C.3.4.11.1 and 3.4.11.2)

The recommended name for this enzyme is aminopeptidase only (Enzyme Commission recommendations, 1972) but since leucine derivatives are some of the commoner substrates used and leucine aminopeptidase is the name generally employed in the literature it will be retained here for clarity.

LAP is widely distributed in human tissues, but chiefly in the small intestine, kidney and liver. Within the liver, LAP is mostly located on the plasma membrane (E.C.3.4.11.2) (Emmelot et al, 1968) but also probably in the cytosol (E.C.3.4.11.1). It catalyses the reaction:

$$\text{RNH.leucine} + H_2O \rightleftharpoons \text{leucine} + \text{R.NH}_2$$

where R may be a variety of groups e.g. a peptide or amino acid. Leucine may be replaced at the N-terminal by a number of other aminoacids although the enzyme is less effective in hydrolysing these.

Like γGT, serum LAP activities may be increased in liver disease, particularly in intra- or extra-hepatic

hepatic obstruction (Kowlessar et al, 1961), but the enzyme is less sensitive than γGT in detecting liver disease (Rosalki, 1975).

Few investigations into the isoenzyme distribution of LAP in health and disease have been undertaken. Normal serum contains a single isoenzyme on starch gel electrophoresis (Lawrence et al, 1960; Kowlessar, Haeffner and Riley, 1961). A further band in the origin region may appear in liver disease of all types. This band corresponds to an ALP band of similar mobility which is also present (Table 1.1) in these diseases. Bile did not possess this band in the region of the origin. Shinkai and Akedo (1972) also found LAP activity in the void volume fraction on Sepharose 4B chromatography of serum from patients with hepatic cancer.

1.5.3. 5'nucleotidase (E.C.3.1.3.5.)

5'NT differs from ALP in that its activity is confined to hydrolysis of nucleotide pentose-5'-phosphate groups only. A typical substrate is adenosine-5'-phosphate (AMP):

$$AMP + H_2O \rightleftharpoons \text{Adenosine} + \text{inorganic phosphate}$$

5'NT is present in a number of human tissues, including liver, thyroid, aorta and bone. In the liver, it can be demonstrated histologically principally on the bile

canalicular membrane (Birns et al, 1962).

Like γGT and LAP, serum levels may be increased in liver disease, particularly obstructive jaundice. However, although the activities of 5'NT show an overall correlation with LAP (Kowlessar, Haeffner, Riley and Sleisenger, 1961) they have been reported to be less sensitive in detecting liver disease (Rosalki, 1975). Bile duct ligation experiments in rats show that, like γGT but unlike ALP, a rise in serum activity precedes a late rise in liver activity. It was not possible to show whether this late rise was due to de novo protein synthesis (Kryszewski et al, 1973). It has been suggested that the late rise in γGT and 5'NT may be due to the proliferation of biliary epithelium which begins two days after bile duct obstruction.

Little information is available concerning isoenzyme patterns of 5'NT. In liver disease, all of it appeared to be in the origin region on starch gel electrophoresis (Kowlessar, Haeffner and Riley, 1961), and in the void volume peak on Sepharose 4B chromatography (Shinkai and Akedo, 1972).

1.6. LIPOPROTEIN X

In obstructive jaundice it has been known for some time that there are derangements of lipid metabolism:

there is a decrease in high density lipoprotein and esterified cholesterol in plasma, coupled with an increase in free cholesterol, phospholipid and low density lipoprotein (Frederickson et al, 1967). The low density lipoprotein is of abnormal composition and is heterogeneous. Whereas some of it contains the apo-B protein of normal low density lipoprotein, some is immunologically distinct and has a separate and distinctive composition. This abnormal lipoprotein has been called lipoprotein X (LPX) (Seidel et al, 1969). LPX has less than 2% protein, partly albumin and partly of the apo-C type, virtually no triglyceride, no esterified cholesterol, considerable amounts of unesterified cholesterol and large quantities of phospholipid comprising 60% of the complex (Seidel et al, 1969; Picard and Veissière, 1970; Seidel et al, 1973).

LPX is not present in the serum of normal individuals. It has been claimed that it appears in the serum of virtually all patients with histologically demonstrable biliary stasis, whether intra- or extrahepatic, and that conversely it is absent in virtually all patients without histologically demonstrable biliary stasis, making it more specific for cholestasis than any other test currently available (Seidel et al, 1970). It cannot, however, distinguish between intra- and extra-

hepatic obstruction.

1.7. AIMS OF THE STUDY

The study was principally concerned with the nature of high mol wt ALP and its clinical evaluation. Other enzymes and LPX were only studied insofar as they contributed to this assessment.

High mol wt ALP might comprise ALP associated with a) protein b) lipid c) carbohydrate or d) membrane fragments containing protein, lipid and carbohydrate. Some of its properties appear to favour the latter hypothesis e.g. a mol wt similar to that of other high mol wt enzymes in serum, known to be principally located on the plasma membrane within the liver (Shinkai and Akedo, 1972). However, this is purely circumstantial evidence. Electrophoretic, catalytic, chromatographic, physical, chemical and immunological investigations were designed to test and develop these alternative hypotheses with special reference to the last hypothesis. The investigations were also designed to test whether high mol wt ALP in serum was heterogeneous, whether and in what respects it resembled the high mol wt ALP in bile, and its similarity or otherwise to the normal liver isoenzyme found in serum. The role of high mol wt ALP in diagnosis was also assessed and combined with the

biochemical studies and with research in a variety of fields to derive a unified theory of the nature of high mol wt ALP and the pathological processes governing its release into serum in liver disease (Chapter 7). Comparisons with similar work carried out by other investigators are discussed where appropriate in the text.

The detailed aims of the study were as follows:

1) To establish whether or not the high mol wt ALP obtained by gel chromatography is identical to any of the bands seen in various media following electrophoresis of serum from patients with liver disease, and to discover which bands in one medium correspond to bands in another medium. In this way, it was hoped that the hitherto confused picture could be clarified. A secondary aim was to identify the simplest, most reproducible, accurate and sensitive method of demonstrating the presence or absence of the high mol wt component and, possibly, of measurement (Chapter 2).
2) To compare the catalytic properties of the high mol wt ALP from serum with those of the liver isoenzyme and also the high and low mol wt isoenzymes in bile. The bone isoenzyme was also studied as a control. Such comparisons, it was hoped, might give some pointer as

to the possible origins of the high mol wt ALP in serum and whether it was closely related to the liver or bile isoenzymes (Chapter 3).

3) To establish the existence or otherwise of high mol wt components of γGT, LAP and 5'NT in serum and bile (for which there is some a priori evidence) since they, like ALP, are membrane-marker enzymes which are released into serum in liver disease. Experiments were designed to test the membrane fragment hypothesis and were conducted on the high mol wt components of all four membrane-marker enzymes in serum and bile in order to test whether or not they formed a single complex. Since LPX has a high mol wt and is also released into the circulation in liver disease, investigations were also directed towards discovering whether any association exists between it and the membrane marker enzymes (Chapter 4).

4) To establish the place of measurements of serum high mol wt ALP in the diagnosis of liver disease. Such an investigation could only be a pilot study, given the limitations of time, but it was hoped that high mol wt ALP could be compared with a broad range of other liver function tests for each of the common categories of liver disease. This might then point the way towards

the most rewarding direction for further research. As a secondary objective it was hoped that the behaviour of high mol wt ALP vis à vis the other liver function tests in disease might shed light on the patho-physiological factors governing its release into the circulation (Chapter 5).

CHAPTER 2

ANALYTICAL METHODS

This chapter concerns the development and validation of the general methods used throughout this thesis for measuring total and high mol wt ALP, γGT, LAP, and 5'NT and for demonstrating the presence or absence of the high mol wt enzymes and LPX. An attempt was made also to identify the different electrophoretic bands seen in various media and relate the bands to those described in the literature (section 1.3.1). Methods used only for particular experiments are described where appropriate in the text.

2.1. MATERIALS

The materials and equipment used are listed below, subdivided for convenience of reference into categories roughly equivalent to the separate chapters of the thesis.

2.1.1. Measurement of total activities of enzymes.

Alkaline phosphatase. 4-Nitrophenyl disodium orthophosphate; 2-amino-2-methyl-propan-1-ol; diethanolamine; Tris (hydroxymethyl) methylamine (AnalaR grade) all from British Drug Houses Chemicals Ltd., Poole, Dorset (BDH).

γGlutamyl transferase. γ-Glutamyl-p-nitroanilide; glycylglycine from Sigma Chemical Co., Poole, Dorset (Sigma).

Leucine aminopeptidase. L-Leucine-p-nitroanilide from Sigma.

5'nucleotidase. Sodium-β-glycerophosphate from BDH. Sodium adenosine 5'monophosphate (from yeast); calf intestinal adenosine deaminase from Sigma.

2.1.2. Isoenzymes

Electrophoretic media. Acrylamide; N:N'-methylene-bis-acrylamide; agarose (for electrophoresis) all from BDH; Cellulose acetate strips from Shandon.
4-26% Polyacrylamide gradient gels from Universal Scientific Ltd., London.

Electrophoresis substrates and dyes. α-Naphthyl acid phosphate (potassium salt); ɣ-glutamyl-β-napthylamide; L-leucyl-β-naphthylamide HCl; 4-aminodiphenylamine diazonium sulphate; Fast Blue BB salt; Fast Violet B salt all from Sigma. Fast Garnet GBC salt from George Gurr Ltd., London. Dimethyl sulphoxide from BDH.

Chromatography. Sepharose 6B from Pharmacia Fine Chemicals, Uppsala, Sweden (Pharmacia). Diethyl amino ethyl cellulose, medium mesh from Sigma. Hibitane (chlorhexidine gluconate 20% w/v) from Imperial Chemical Industries, Ltd., Macclesfield, Cheshire.

2.1.3. Purification and Kinetics

Protamine sulphate from salmon (Grade II); Coomassie Brilliant Blue G; L-leucine; L-phenylalanine all from Sigma. L-(+)-homoarginine-HCl (99+%) from Aldrich

Chemical Co. Ltd., Gillingham, Dorset.

2.1.4. Physical and Biochemical Properties

Urease (from jack-beans); catalase (from bovine liver) both from Sigma. Neuraminidase (from vibrio comma (cholerae)) from Hoechst Pharmaceuticals, Hounslow (Hoechst). Urea (AnalaR); Butan-1-ol (AnalaR); di-iso-propyl ether; sodium dodecyl sulphate all from BDH. Triton X-100 (octylphenoxypolyethoxyethanol) from Sigma. Bacto-agar from Difco Laboratories, Detroit, Michigan, U.S.A. Nigrosin from George Gurr Ltd., London. Amido black 10B stain from Corning ACI, Palo Alto, California. Antisera against human lipoprotein X, β lipoprotein, IgG, IgA and IgM from Hoechst.

2.1.5. Slow band alkaline phosphatase

Human γglobulin from Sigma. Human albumin; anti-sera against human IgG, IgA and IgM from Hoechst.

2.1.6. Equipment

LKB 8600 reaction rate analyser; SP1800 ultraviolet recording spectrophotometer (from Pye Unicam); Sequential Multiple Analyzer plus Computer (Technicon). Electrophoresis tank for vertical slab gels (Shandon); electrophoresis tank for horizontal gels (Shandon); Shandon power supply (up to 400 volts); electrophoresis tank for polyacrylamide gradient gels and power pack

(Universal Scientific Ltd.); Vitatron densitometer. Chromatography columns (Pharmacia); LKB 8300 Uvicord II spectrophotometer; LKB biocal recorder; LKB 7000 ultrorac fraction collector; LKB 2115 multiperpex pump; LKB Conductolyzer Type 5300B; disposable plastic syringes; gauze Inco tissues (Robinsons, Chesterfield).

2.2. MEASUREMENT OF TOTAL ENZYME ACTIVITIES

2.2.1. Alkaline phosphatase

In serum, ALP activity was measured on the Technicon Sequential Multiple Analyzer plus Computer (SMAC) using as substrate 10 mmol/l p-nitrophenyl phosphate in 0.6 mol/l 2-amino-2-methyl-1-propanol buffer pH 10.2 containing 0.5 mmol/l magnesium chloride (Bowers and McComb, 1966). The between batch coefficient of variation was 2.5%.

In column effluents, to obtain greater sensitivity, a continuous monitoring (kinetic) method was used to measure ALP activity (McComb and Bowers, 1972). After mixing the column effluent with an equal volume of 1.8 mol/l diethanolamine buffer pH 10.2 containing 1 mmol/l magnesium chloride, p-nitrophenyl phosphate was added to give a final concentration of 14 mmol/l. The reaction was monitored at 410 nm at 37^{o}C in an LKB reaction rate analyser. A molar extinction coefficient of 18 800 for p-nitrophenol

was used in the calculation.

2.2.2. γGlutamyl transferase

A modification of the method of Rosalki and Tarlow (1974) was used for the measurement of γGT activity in serum and in column effluents.

50 µl serum was mixed with 1.0 ml Tris-glycyl-glycine buffer pH 8.5. The reaction was started with 0.1 ml γ-glutamyl-p-nitroanilide as substrate, dissolved in 0.5 mol/l HCl. The concentrations of the reagents were such as to give the following final reaction conditions:

4.5 mmol/l γglutamyl-p-nitroanilide

100 mmol/l Tris

50 mmol/l glycylglycine

pH 8.0

The reaction was monitored at 410 nm at 37°C in an LKB 8600 reaction rate analyzer. A molar extinction coefficient of 9900 for p-nitroaniline was used in the calculations.

A similar method was used for the measurement of γGT in column effluents except that 0.5 ml column effluent was mixed with 0.5 ml Tris-glycylglycine buffer the composition of which had been adjusted to give the same final reaction conditions.

2.2.3. Leucine aminopeptidase

A modification of the method of Szasz (1967) was used for the measurement of LAP activity in serum and in column effluents.

50 µl serum was mixed with 1.0 ml 0.1 mol/l Tris-HCl buffer pH 7.7. The reaction was started with 50 µl L-leucine-p-nitroanilide dissolved in 0.25 mol/l HCl. The concentrations of the reagents were such as to give the following final reaction conditions:

0.8 mmol/l L-leucine-p-nitroanilide

0.1 mol/l Tris-HCl buffer pH 7.5

The reaction was monitored at 410 nm at 37°C in an LKB 8600 reaction rate analyser. A molar extinction coefficient of 9900 for p-nitroaniline was used in the calculations.

A similar method was used for the measurement of LAP in column effluents except that 0.5 ml column effluent was mixed with 0.5 ml buffer the composition of which had been adjusted to give the same final reaction conditions.

2.2.4. 5'nucleotidase

5'NT activity in serum and in column effluents was measured according to the method of Belfield and Goldberg (1969). Using 5'adenosine monophosphate (5'AMP) as substrate and adenosine deaminase (ADA) as coupling enzyme,

the principle of the reaction was as follows:

$$5'AMP + H_2O \xrightarrow{5'NT} \text{Adenosine} + \text{inorganic phosphate}$$

$$\text{Adenosine} + H_2O \xrightarrow{ADA} \text{Inosine} + NH_3$$

βGlycerophosphate was used to inhibit the non-specific phosphatase activity. The reaction was started by the addition of 5'AMP and the decrease in absorbance at 265 nm due to the conversion of adenosine to inosine was monitored continuously at 37°C against a reagent blank on an SP1800 spectrophotometer. The final reaction concentrations were as follows:

0.1 mol/l Tris-HCl buffer pH 7.9

10 mmol/l $MgCl_2$

15 mmol/l sodium βglycerophosphate

2 mg/l adenosine deaminase

0.1 mmol/l 5'AMP

For the calculations, an extinction coefficient of 8.0 x 10^6 $cm^2 mole^{-1}$ was assumed for the difference between the absorbance of 5'AMP and inosine at 265 nm under the conditions of the reaction.

2.3. ELECTROPHORESIS AND LOCALISATION OF ISOENZYMES

2.3.1. Alkaline phosphatase

Polyacrylamide gel. Electrophoresis of sera in uniform vertical polyacrylamide gel slabs was performed according to a modification (Warwick et al, 1972) of the method of

Kaplan and Rogers (1969). The buffer used throughout was 0.37 mol/l Tris-borate pH 9.5 containing 0.5 mmol/l $MgCl_2$ and electrophoresis was at a constant voltage of 300 volts. For the weaker gels, a retaining strip of 7% polyacrylamide gel was used at the lower edge of each gel slab since otherwise these gels did not have enough structural strength to remain in place between the glass plates. For similar reasons, after staining, instead of being stored in the wet state in polythene wrappings, the weak gels were stored by drying them between a glass plate and filter paper backing.

Electrophoresis of sera in 4-26% polyacrylamide gradient gels, pre-equilibrated with the same buffer, was at 100v for 24 hours at 4°C.

Qualitative localisation of the isoenzyme bands was achieved by immersion in 0.75 mmol/l α-naphthyl acid phosphate in buffer for 20 min followed by immersion in 0.75 mmol/l α-naphthyl acid phosphate/0.5 g/l 4-aminodiphenylamine diazonium sulphate in buffer overnight at 4°C in the dark. Semi-quantitative localisation used the second substrate-dye solution only for 5 hours at room temperature in the dark. The gels were cleared using 7% acetic acid.

Agarose gel. Various concentrations of agarose gel in

0.2 mol/l Tris-borate buffer pH 10.0 were prepared by melting the agarose in buffer in a bath of boiling water. 10 ml of the solution was poured onto a horizontal 10 x 7.5 cm plate and allowed to set at room temperature. Three 1 x 10 mm slots in each gel were cut, sera were applied to the slots and subjected to electrophoresis in the same buffer at a constant voltage of 300v for 3-4 hours at 4°C. The staining procedure was the same as that described for polyacrylamide gel.

Cellulose acetate. Cellulose acetate strips (7.5cm x 14.5 cm) were pre-soaked in 50 mmol/l Tris-barbital buffer pH 8.8. Sera were applied using a 1 µl applicator and subjected to electrophoresis in the same buffer at a constant voltage of 200v for 1½ hours.

Two alternative methods were used for staining cellulose acetate strips. The first method used essentially the one-step staining method described for polyacrylamide and agarose gels, except that, instead of being immersed in the staining solution, the strip was imprinted for 2 hours at room temperature face down on a glass plate covered with a thin film of the staining solution. An alternative technique, based on the method of Burlina and Galzigna (1976) was also investigated because of the problems of diffusion in cellulose acetate.

During electrophoresis, a solution of 2% agarose in 0.37 mol/l Tris-borate buffer pH 9.5 containing 0.5 mmol/l magnesium chloride was prepared by melting the agarose in the buffer in a bath of boiling water, and cooled to 37°C. An equal volume of a solution containing 4 g/l α-naphthyl acid phosphate and 4 g/l 4-aminodiphenylamine diazonium sulphate was prepared in the same buffer and warmed to 37°C. Immediately before use, the two solutions were combined, mixed thoroughly and poured onto a tray to set at room temperature. The cellulose acetate strip was imprinted on the gel face down and incubated in the dark at room temperature for 1½ hours.

2.3.2. γGlutamyl transferase

The electrophoretic medium which provided the greatest sensitivity in the detection and localisation of γGT isoenzymes was found to be polyacrylamide gel. Uniform 2.5% and 7% gels and 4-26% gradient gels were used for the electrophoretic separation of the isoenzymes. A gel buffer of 0.72 mol/l Tris-HCl buffer pH 8.9 and a tank buffer of 0.05 mol/l Tris-glycine buffer pH 8.3 were employed (Azzopardi and Jayle, 1973). Electrophoresis was for 2-3 hours at a constant voltage of 300v for the uniform gels and overnight at 100v for gradient gels.

A substrate solution was prepared by dissolving

25 mg γ-glutamyl-β-naphthylamide in 0.25 ml dimethyl sulphoxide and 0.25 ml 1 mol/l NaOH and mixing this solution with 50 ml 50 mmol/l Tris/25 mmol/l glycyl-glycine buffer pH 8.9 (Rosalki, personal communication). The gel was immersed in this solution for 2 hours at room temperature. It was then transferred to a staining solution of 2 g/l Fast Garnet GBC in 0.25 mol/l Tris-maleate buffer pH 6.2 for 2 hours at room temperature. Fast Garnet GBC was found to give greater sensitivity than the other dyes tested (Fast Blue BB, Fast Violet B and 4-aminodiphenylamine diazonium sulphate). The gels were cleared using 7% acetic acid.

2.3.3. Leucine aminopeptidase

Electrophoresis in polyacrylamide gel was carried out exactly as described for γGT.

The substrate solution was prepared by dissolving 20 mg L-leucyl-β-naphthylamide in 2 ml 0.01 mol/l HCl and mixing this solution with 50 ml 0.02 mol/l phosphate buffer pH 7.0 (Goldbarg and Rutenburg, 1958). The gel was immersed in this solution for 2 hours at room temperature and then transferred to a staining solution of 2 g/l Fast Garnet GBC in 0.25 mol/l Tris-maleate buffer pH 6.2 for 2 hours. 7% acetic acid was again used for clearing the gels.

2.4. LIPOPROTEIN X

Immunoelectrophoresis was carried out by a modification (Seidel et al, 1970) of the Scheidegger technique (1955). 1% unpurified agar in 0.05 mol/l barbital buffer pH 8.6 was used on 10 x 8 cm horizontal plates. 4 µl sample was applied to 2 mm diameter wells and subjected to electrophoresis at a constant voltage of 200v for 2 hours. 1 mm wide troughs were cut equidistant between the wells and filled with LPX antiserum which was allowed to diffuse at room temperature overnight. After the formation of any precipitin arcs, the gels were washed for 2 days in saline, stained for protein using nigrosin which was found to be the most sensitive stain for this purpose, and cleared in methanol:acetic acid:water (9:2:9). The gels were also stained for enzyme activity using the methods outlined in section 2.3.

A precipitin spur on the cathodal side of the wells indicated that LPX was present. The LPX antiserum used was raised against Apo-C but is known to cross-react with other plasma proteins including some lipoproteins. However, all these cross-reacting proteins and lipoproteins migrate towards the anode under the conditions of the assay and only LPX migrates towards the cathode. In practice, therefore, the method is specific.

An attempt was also made to detect LPX by the Seidel (1971) technique which, following electrophoresis, involves *in situ* precipitation of LPX by a drop of antiserum placed on the cathodal side of the wells. However, this method was found to be less reliable than the Scheidegger technique because the precipitate sometimes redissolved.

Quantitation of LPX by Laurell rocket electroimmunoassay (Laurell, 1966) proved to be unsatisfactory. Irregularly shaped rockets, often with ill-defined peaks, were obtained. Moreover the height of the rocket did not appear to correlate with the presence, amount or complete absence of LPX using the Scheidegger technique. When the experiment was repeated using βlipoprotein antiserum instead of LPX antiserum in the gel, the appearance, following prolonged electrophoresis, of similar rockets migrating towards the cathode suggested that under these conditions some βlipoprotein might have migrated towards the cathode. This would then have cross-reacted with the LPX antiserum in the original gel. Such a hypothesis would explain the appearance of apparent "LPX" rockets in sera from normal subjects possessing no serum LPX according to the Scheidegger technique.

In summary, the evidence indicated that the

Scheidegger technique was the most suitable method for detecting LPX in serum and this method was accordingly chosen.

2.5. MOBILITY OF ALKALINE PHOSPHATASE ISOENZYMES DURING ELECTROPHORESIS IN VARIOUS MEDIA

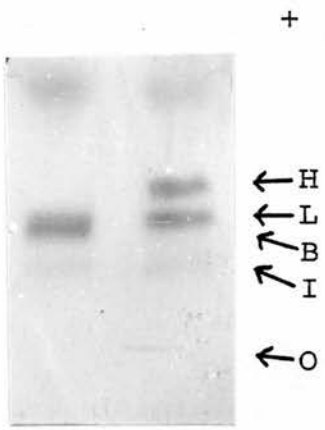
Sera containing liver, bone, intestinal and high mol wt ALP in varying proportions were subjected to electrophoresis in the following media according to the methods outlined in paragraph 2.3.1.

1) cellulose acetate
2) 1%, 2% and 2.2% agarose gels
3) 2%, 2.5%, 3% and 7% polyacrylamide gels
4) 4-26% polyacrylamide gradient gel

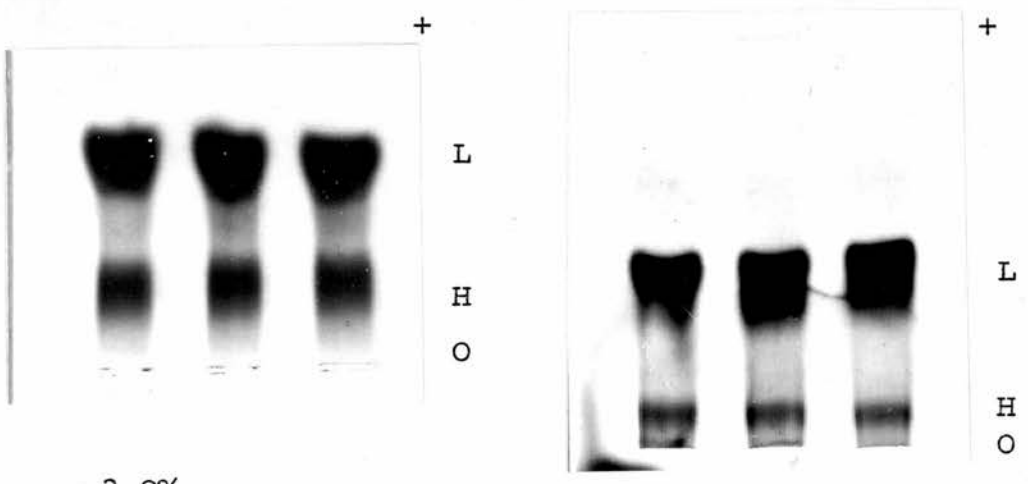
The gels and cellulose acetate strips were stained for ALP by the methods described in paragraph 2.3.1. Examples of the isoenzyme patterns obtained in the various media are shown in Fig. 2.1. The positions of the isoenzyme bands in these media are shown schematically in Fig. 2.2.

Cellulose acetate. Cellulose acetate separates proteins purely on the basis of charge. In this medium high mol wt ALP migrated ahead of the liver isoenzyme (Fig. 2.2 (a)). High mol wt ALP would therefore appear to be more highly charged than any of the isoenzymes commonly found in serum.

Figure 2.1. Positions of ALP isoenzyme bands after electrophoresis of sera in various electrophoretic media. O, origin; I, intestinal isoenzyme; B, bone isoenzyme; L, liver isoenzyme; H, high mol wt ALP. Electrophoresis was towards the anode in every case.

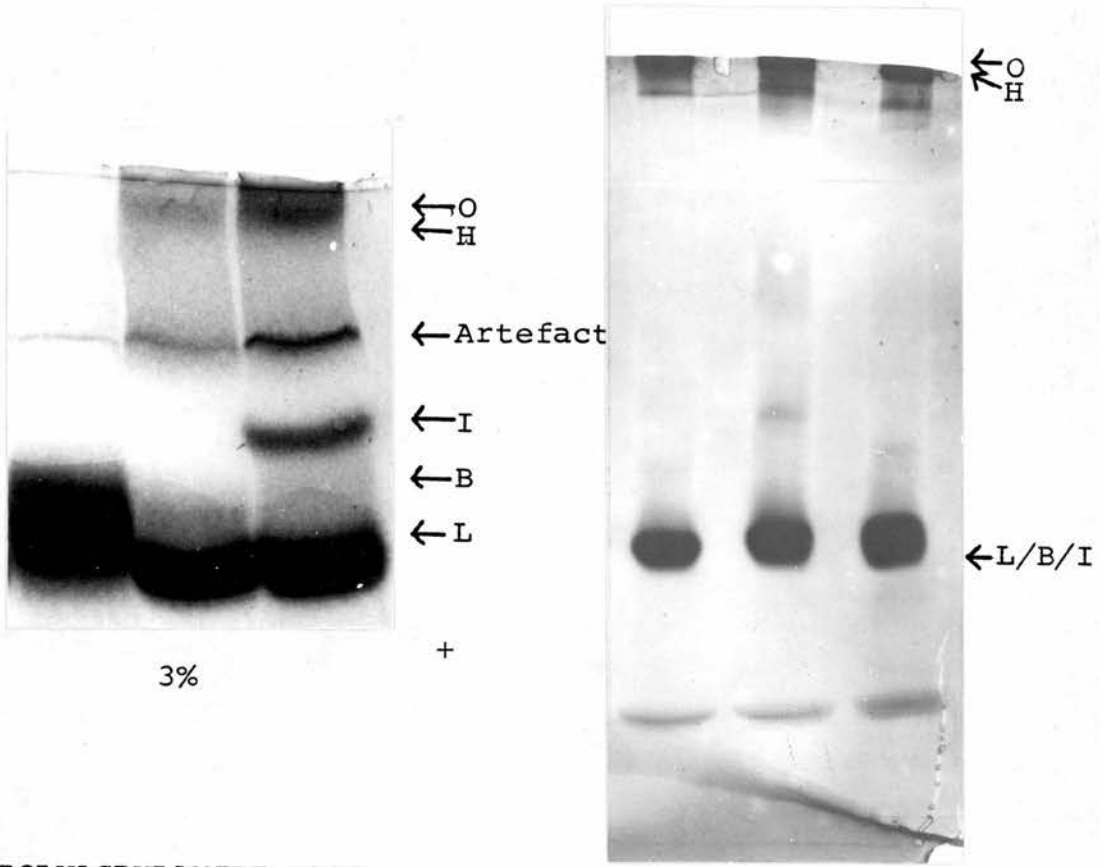


(a) CELLULOSE ACETATE



(b) AGAROSE GELS

Figure 2.1. (continued).



(c) POLYACRYLAMIDE GELS

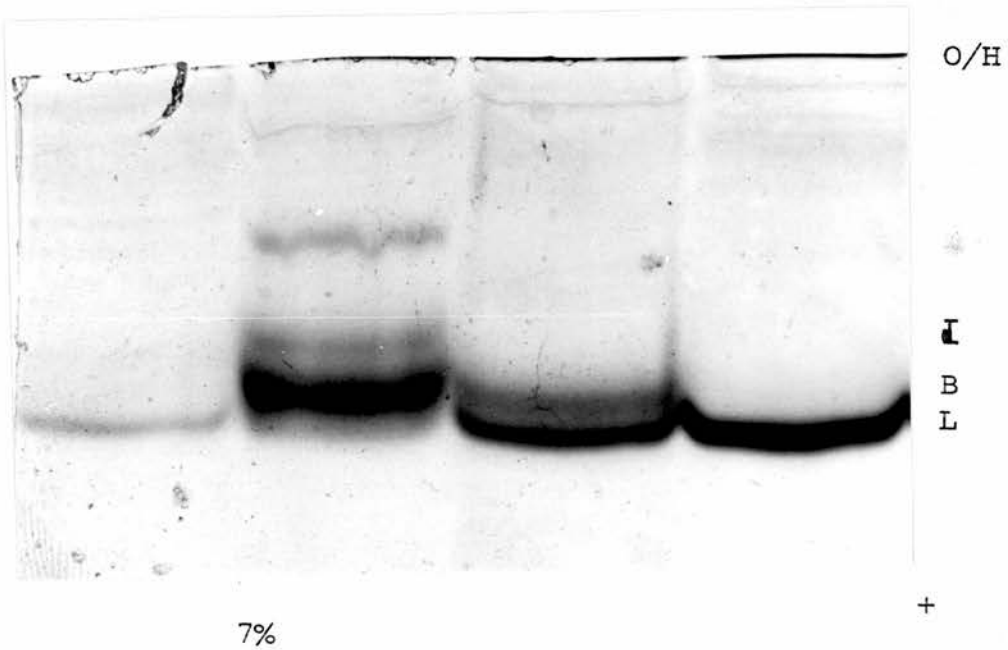
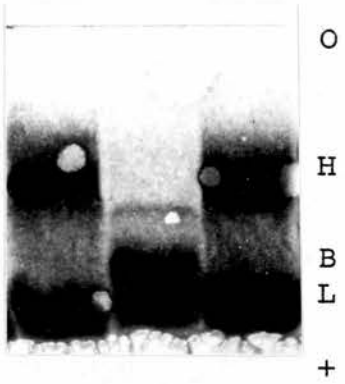
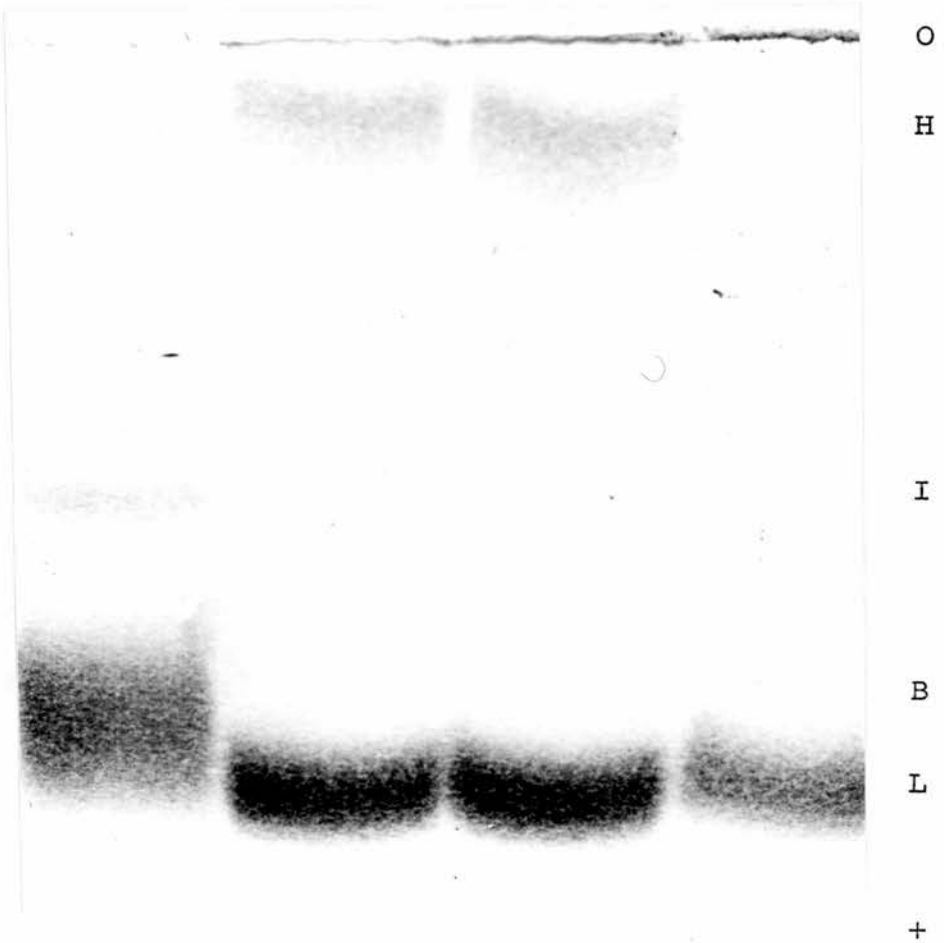


Figure 2.1. (continued).

(c) POLYACRYLAMIDE GELS

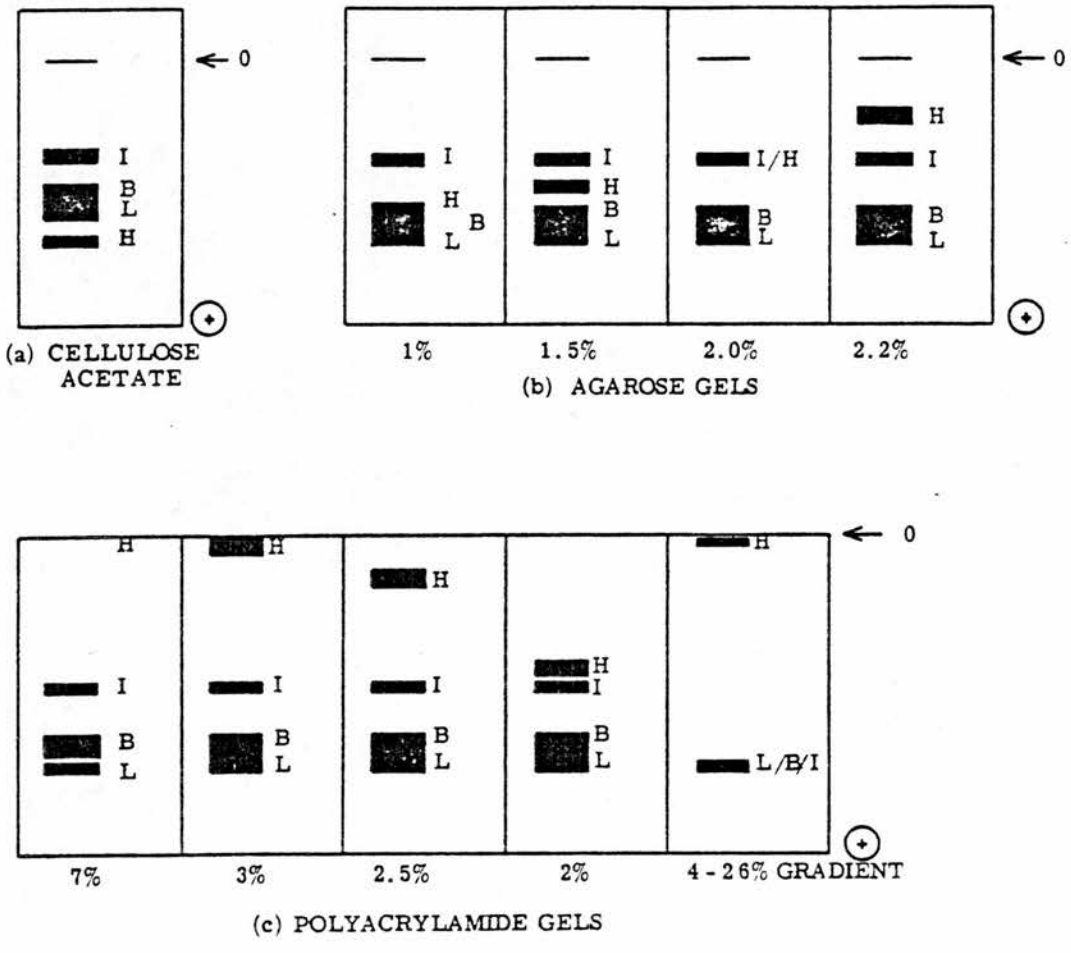


2%



2.5%

Figure 2.2. Schematic representation of positions of ALP isoenzyme bands after electrophoresis of sera in various electrophoretic media. Abbreviations as in Fig 2.1.



Agarose gel. It is generally assumed that agarose gel also separates proteins purely on the basis of charge. However, in 1% agarose, the concentration commonly employed, the high mol wt ALP migrated closely behind the liver isoenzyme, suggesting that some retardation was taking place owing to the large molecular size of the component. This hypothesis gains support from the evidence that increasing concentrations of agarose progressively retarded high mol wt ALP (Fig 2.2. (b)). A 2.2% agarose concentration was found to be optimal since at this concentration high mol wt ALP was separated from all the other alkaline phosphatase isoenzymes.

Polyacrylamide gel. Polyacrylamide gels separate proteins partly on the basis of charge and partly on the basis of molecular size. This is illustrated by the observation that high mol wt ALP was found to be excluded from the 7% gel matrix and remained at the origin, but it penetrated progressively further into 3%, 2.5% and 2% gels respectively (Fig 2.2.(c)). In a 4-26% polyacrylamide gradient gel, proteins are separated purely on the basis of molecular size since electrophoresis is to equilibrium. In this gel, high mol wt ALP migrated only a short distance into the gel matrix. A 2.5% polyacrylamide gel was found to be optimal since this gel allowed full

penetration of high mol wt ALP into the gel matrix and good separation from the other isoenzymes, while retaining sufficient structural strength.

2.6. QUANTITATION OF ALKALINE PHOSPHATASE ISOENZYMES BY ELECTROPHORESIS

Cellulose acetate was found to be an unsuitable medium for quantitation of ALP isoenzymes because of problems related to diffusion. When the strips were stained by imprinting on glass plates overlaid with a film of staining solution, diffusion took place laterally within the strip and outwards into the staining solution. The technique of imprinting on agarose gel appeared to diminish the first of these effects but had little effect on the second. Indeed, it aggravated the problem because the lower mol wt isoenzymes appeared to diffuse much more readily into the agarose gel than did the high mol wt component, thereby giving rise to larger inaccuracies in measurement.

For this reason only agarose and polyacrylamide gels were investigated with regard to their suitability for measurement of the high mol wt component. In each case quantitation was achieved by scanning the stained gels using a Vitatron densitometer followed by measurement of the areas under the peaks. In both types of gel,

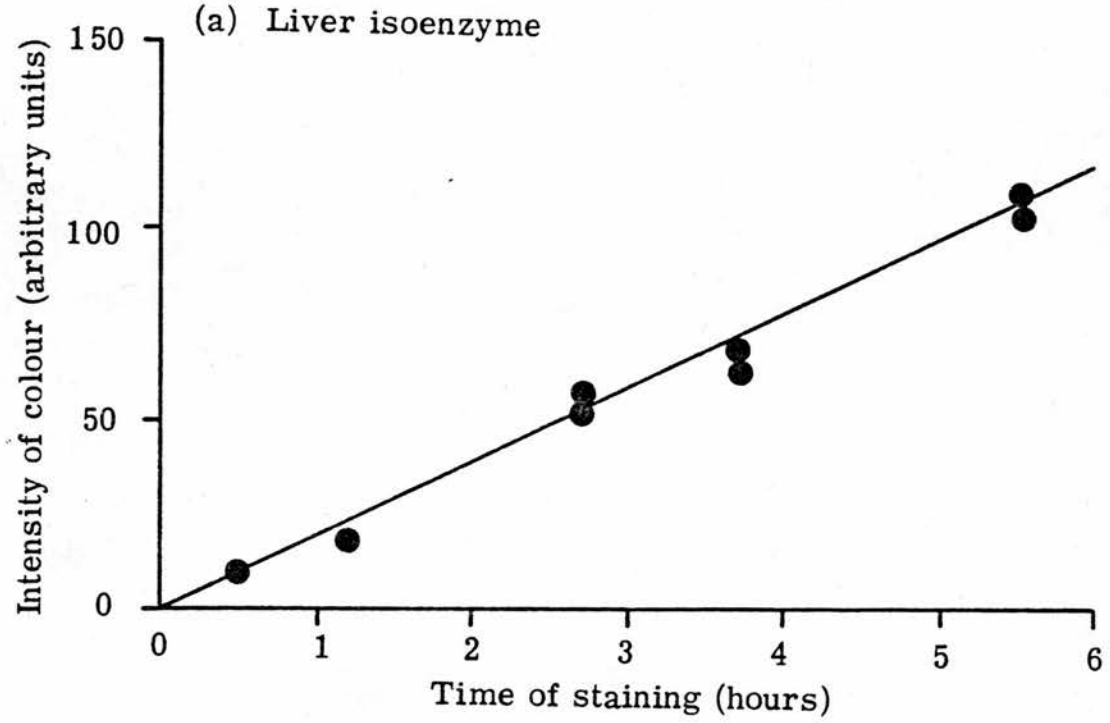
non-specific dye uptake gave a faint yellow band in the region of the gel between the high mol wt band and the liver isoenzyme band. This did not coincide with any isoenzyme band and did not interfere with measurement.

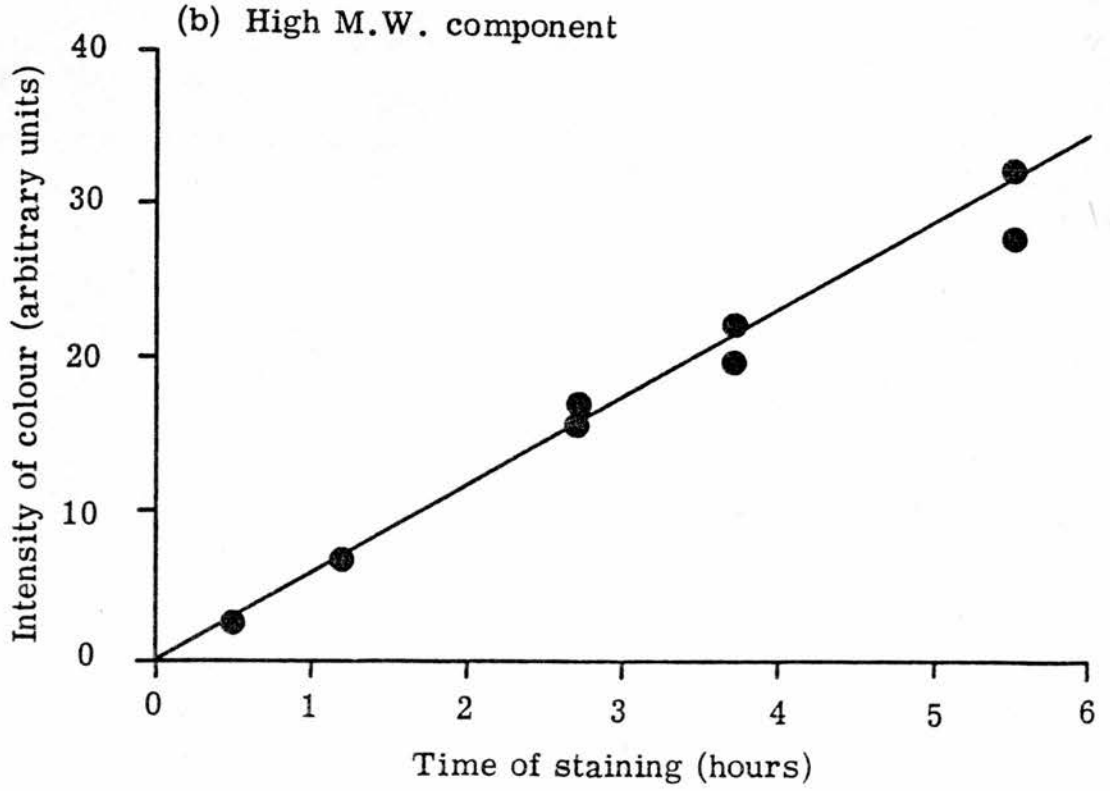
2.6.1. Staining time

In order to select the optimum length of time for the one-step staining procedure (section 2.3.1.), linearity of colour development with time was assessed. After electrophoresis of several identical aliquots of a serum containing liver and high mol wt ALP, the gel was immersed at room temperature in substrate-dye solution for various lengths of time. At the end of each time period a slice of gel corresponding to one aliquot of serum was immersed in 7% acetic acid to stop the reaction and clear the colour background. After clearing, the isoenzyme bands were measured by scanning.

For 2.5% polyacrylamide gel, the increase in colour developed (expressed as area under the peak) with time was linear up to 6 hours (Fig 2.3.). The amount of high mol wt ALP present, expressed as the calculated percentage of the total ALP activity, was found to be constant for a staining time between 3 and 6 hours. 4 hours was chosen as the optimal staining time. For 2.2% agarose, similar considerations led to the adoption of 4 hours as the

Figure 2.3. Development of colour (measured by scanning densitometry) with time following electrophoresis on 2.5% polyacrylamide gel.





optimal staining time.

2.6.2. Dilutional linearity of staining reaction

A serum containing liver and high mol wt ALP was diluted serially in 0.15 mol/l saline. These dilutions were subjected to electrophoresis in 2.5% polyacrylamide gel and 2.2% agarose gel. The gels were stained for 4 hours, cleared in 7% acetic acid and scanned. The activities of the liver and high mol wt components of ALP were calculated for each dilution using the formula:

$$\text{Activity of isoenzyme} = \frac{\text{area under isoenzyme peak x activity of dilution}}{\text{total area under both peaks}}$$

Fig. 2.4. demonstrates that in both types of gel there is a linear relationship between the activities of both liver and high mol wt ALP and dilution. However, there is slightly more scatter of the individual data points in the case of 2.2% agarose gel. In both gels, there is some evidence that there may be some degree of non-linearity at low activities since the regression lines do not pass through the origin.

2.6.3. Precision

The within-run coefficients of variation for the percentage of high mol wt ALP in serum were 3.7% (N=10, Mean:28.8%) for 2.5% polyacrylamide gel and 4.6% (N=9, Mean:31.9%) for 2.2% agarose gel.

Figure 2.4. Relationship between ALP activity, calculated by scanning densitometry, and dilution in a serum containing liver and high mol wt ALP.

(a) 2.2% agarose.

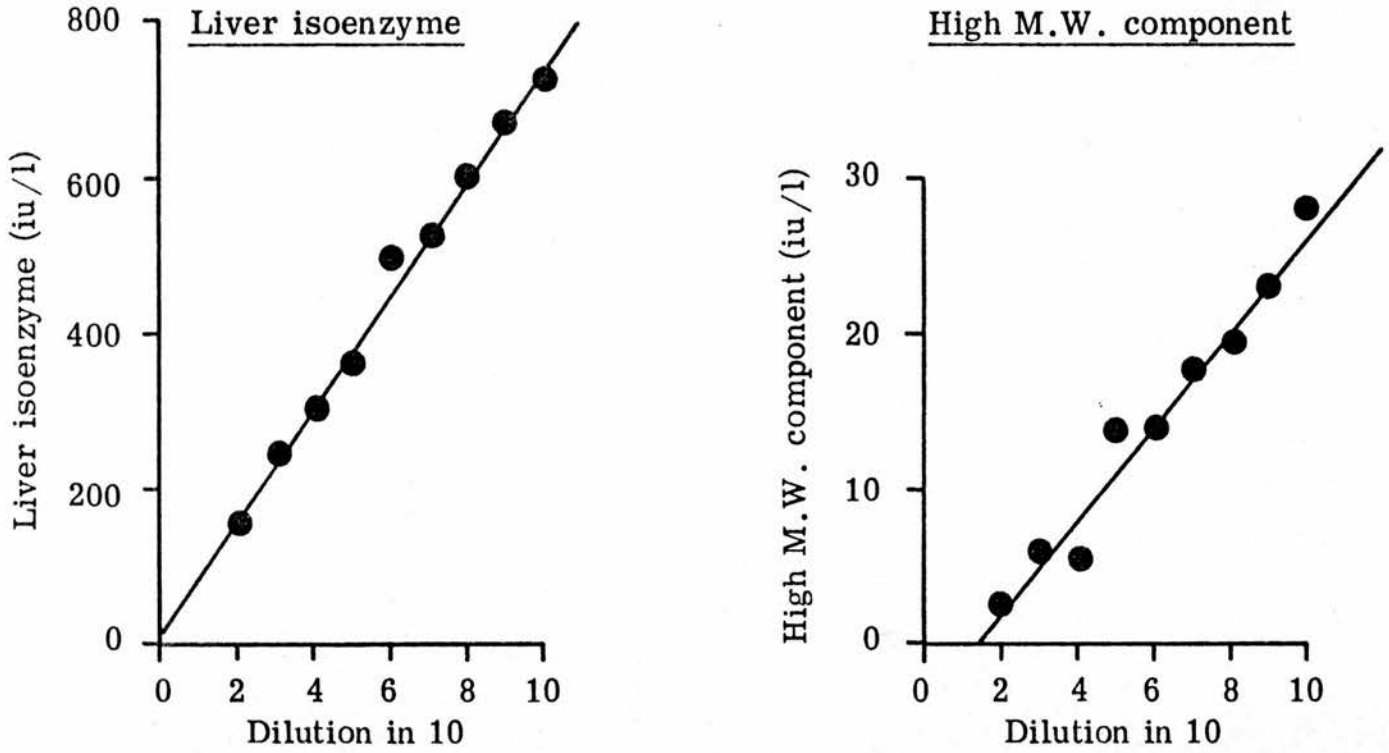
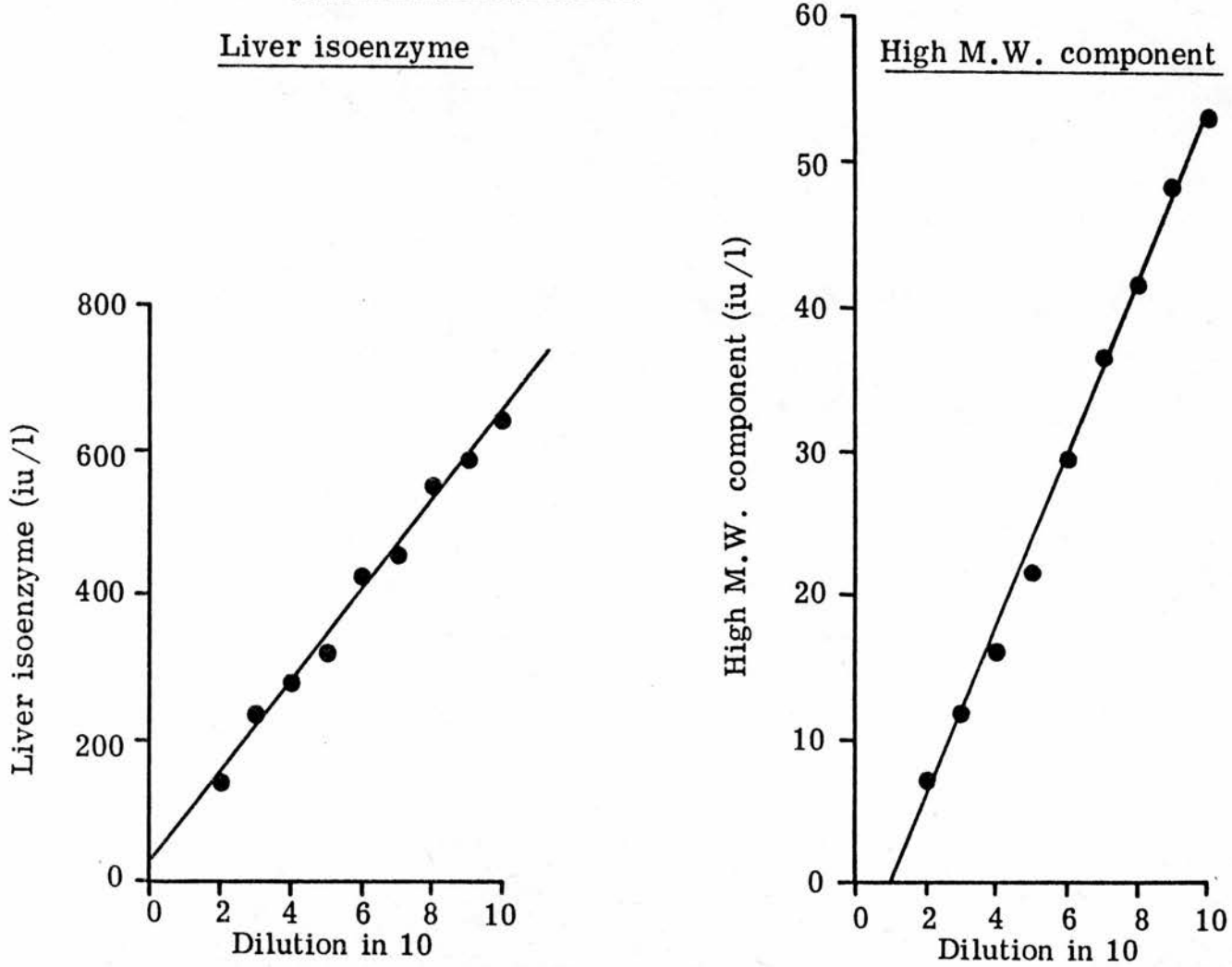


Figure 2.4. (continued).

(b) 2.5% polyacrylamide



The between-run coefficients of variation for the percentage of high mol wt ALP in serum, based on analysis of duplicates, were 14.6% (N=9, Mean:9.7%, Range: 2.4-17.7%) for 2.5% polyacrylamide gel and 27.5% (N=14, Mean:16.4%, Range: 2.7-40.5%) for 2.2% agarose gel.

2.6.4. Comparisons between high molecular weight alkaline phosphatase estimates obtained by electrophoresis in different media

Electrophoresis of 6 sera was carried out in various media and the high mol wt ALP estimated by scanning densitometry according to the methods described in section 2.3.1. The sera contained liver, bone, intestinal and high mol wt ALP in varying proportions. The results are shown in Table 2.1. With the exception of the agar imprinting technique used with cellulose acetate strips, roughly comparable results for the different methods were obtained. The overlay technique gave results which were about twice as high as those with other methods. This was attributed to the slower rate of diffusion of the high mol wt ALP into the agarose, leaving more of this enzyme on the strip which was subsequently scanned for quantitation of the isoenzymes: the overlay, on the other hand, showed denser staining in the region of the lower mol wt ALP. In general, the lack of close agreement

TABLE 2.1.

Percentage of high mol wt ALP in six sera, estimated by scanning densitometry, following electrophoresis and staining in three media

Serum	Total ALP activity (iu/l)	High mol wt ALP (% total)			
		2.2% agarose gel	2.5% polyacrylamide gel	Cellulose acetate	
				Thin-film staining	Agar template staining
1	144	26	20	22	48
2	304	32	30	32	56
3	544	17	21	33	49
4	216	24	17	15	50
5	568	3	4	14	15
6	520	8	5	14	24

between the results obtained in the various media reflects the poor precision, and suggests that the electrophoretic techniques are best regarded as semi-quantitative.

2.7. QUANTITATION OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE BY SEPHAROSE 6B CHROMATOGRAPHY

2.7.1. Assay procedure

2ml serum was applied to a 95 x 2.5cm column containing Sepharose 6B equilibrated with 0.1 mol/l Tris-HCl buffer pH 7.7 containing 50 mmol/l NaCl. The serum was eluted overnight at 4°C using the same buffer at a flow rate of 28 ml per hour, and 5 ml fractions were collected. The protein content of the eluate was measured by continuous monitoring at 280 nm. The ALP activity in each fraction was measured by the method outlined in paragraph 2.2.1. A typical sample elution profile is shown in Fig 2.5. The peak corresponding to high mol wt ALP eluted in the void volume. The low mol wt peak contained the liver, bone and intestinal isoenzymes.

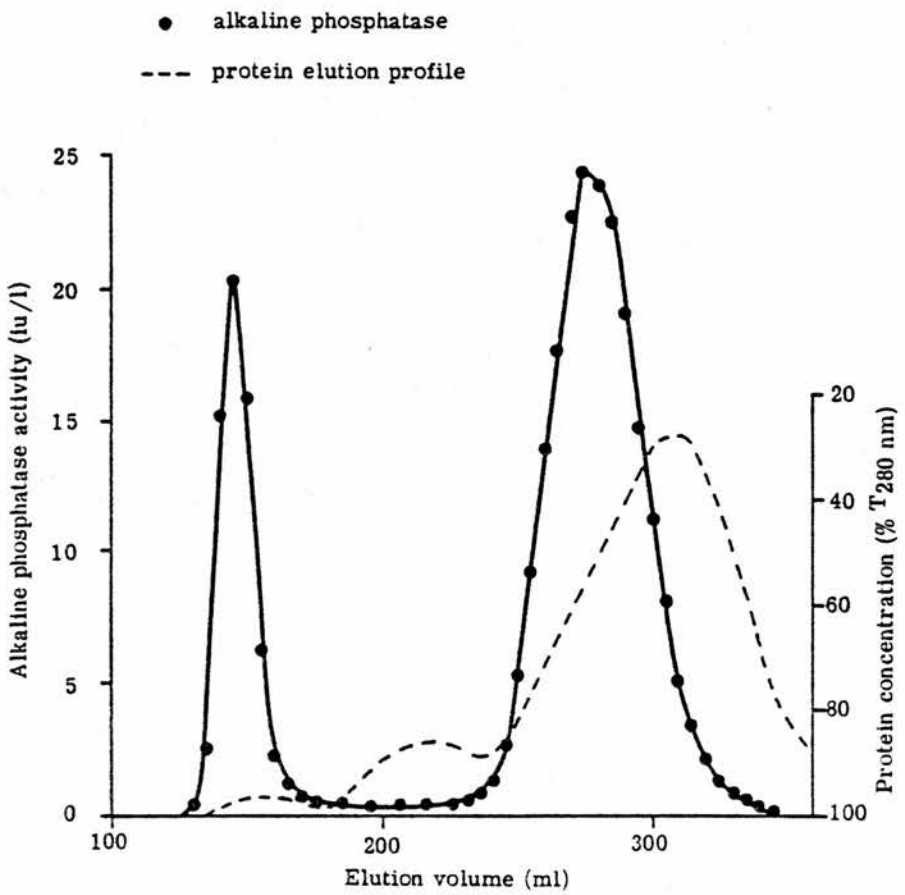
The activities of all the fractions in each peak were summed and the activity of the high mol wt component was calculated using the formula:

$$\text{Activity of high mol wt component (iu/l)} = \frac{A}{A + B} \times \text{activity of serum (iu/l)}$$

where A = sum of activities of fractions in high mol wt peak

B = sum of activities of fractions in low mol wt peak.

Figure 2.5. Elution profile of a serum containing liver and high mol wt ALP, obtained by Sepharose 6B chromatography. The bone and intestinal isoenzymes also eluted in the low mol wt peak.



2.7.2. Validation of method

The mean recovery of ALP activity from the column was 97.4% (N=7, Range 78-115%). Since the method was time-consuming and tedious to perform, it was not practicable to calculate the precision for a large number of samples. However, duplicate analyses of the 4 samples shown in Table 2.2 suggested that the reproducibility was acceptable. The between-batch coefficient of variation for measurement of ALP activities in the fractions was 1.3% (N=46).

TABLE 2.2.

Reproducibility of estimates of high mol wt ALP (expressed as a percentage of total ALP activity in serum) obtained by Sepharose 6B chromatography

Serum	High mol wt ALP (%)	
	First analysis	Second analysis
1	21.4	19.7
2	21.3	19.6
3	30.3	32.5
4	12.6	13.0

2.8 COMPARISON BETWEEN ESTIMATES OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE BASED ON a) SEPHAROSE 6B CHROMATOGRAPHY AND b) 2.5% POLYACRYLAMIDE GEL ELECTROPHORESIS

Two sera, A and B (Table 2.3.), containing liver and high mol wt ALP only were analysed by Sepharose 6B chromatography and by electrophoresis in 2.5% polyacrylamide gel. The electrophoretic method gave much lower estimates of high mol wt ALP, a discrepancy of approximately four-fold.

TABLE 2.3.

Comparison between estimates of high mol wt ALP, expressed as a percentage of total ALP activity in serum, obtained by Sepharose 6B chromatography using a) p-nitrophenyl phosphate (pNPP) and b) α-naphthyl acid phosphate (αNP) as substrates, and by electrophoresis on 2.5% polyacrylamide gel using α-naphthyl acid phosphate as substrate

Method	Substrate	% high mol wt ALP			
		Serum A	Serum B	Serum C	Serum D
Sepharose 6B	pNPP	21.4	19.5		8.0
	αNP			33.3	1.5
Electrophoresis	αNP	5.6	4.5	28.5	

Since the gel chromatography method used p-nitrophenyl phosphate as substrate whereas the electrophoretic method used αnaphthyl phosphate as substrate it was thought that

this discrepancy might be due to different substrate specificities of liver and high mol wt ALP. A quantitative assay was therefore set up, using αnaphthyl phosphate as substrate, to measure the activities of the Sepharose 6B column eluate. This assay reproduced as closely as possible the reaction conditions of the substrate solution used in polyacrylamide gel staining. Thus, the final reaction conditions of this assay were as follows:

0.75 mmol/l α-naphthyl acid phosphate
0.37 mol/l Tris-borate buffer pH 9.5
0.5 mmol/l $MgCl_2$

The increase in absorbance was monitored at 340 nm at 37°C. The between-batch coefficient of variation for this method was 6.4% (N=30).

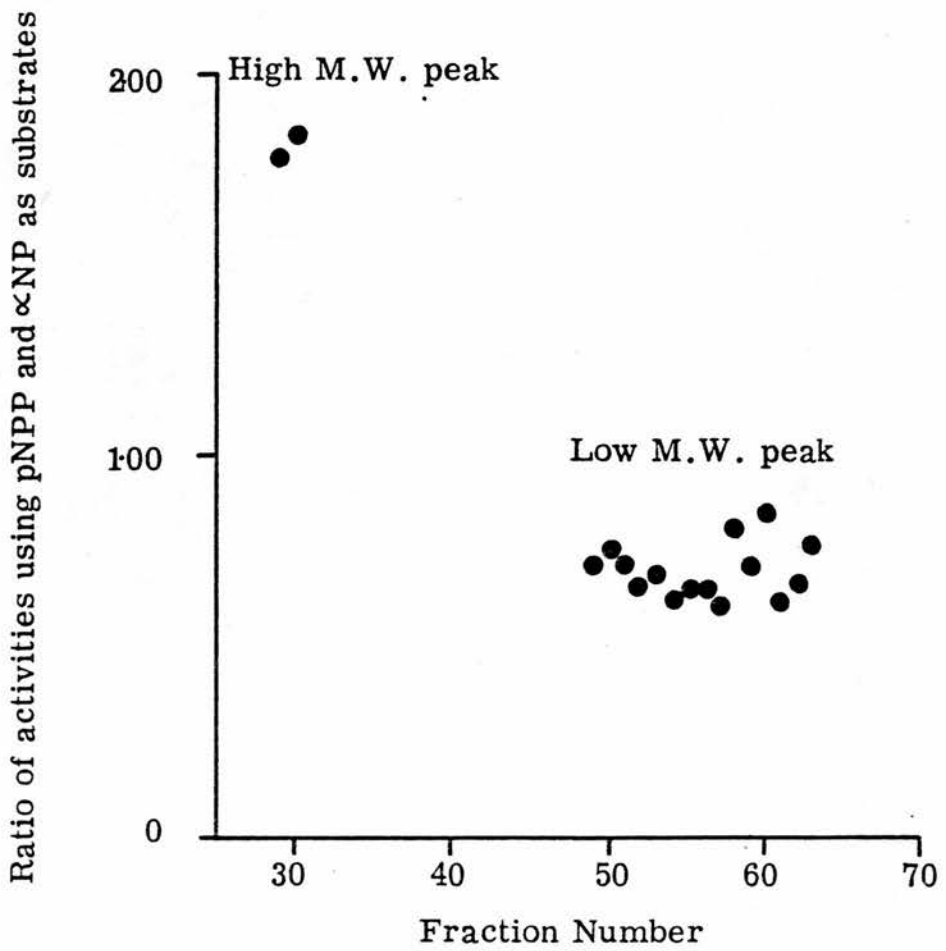
When serum C(Table 2.3.) was analysed by Sepharose 6B chromatography and by polyacrylamide gel electrophoresis, using αnaphthyl phosphate as substrate on both occasions, results in much closer agreement with each other were found.

These observations suggest that liver and high mol wt ALP differ in their specificities for p-nitrophenyl phosphate and α-naphthyl phosphate under the conditions of assay. In order to confirm this, serum D(Table 2.3.) was applied to the Sepharose 6B column and the activity in each fraction was measured using a) p-nitrophenyl phosphate as substrate and b) α-naphthyl phosphate as substrate. Much

lower results were obtained for high mol wt ALP using α-naphthyl phosphate (again a four-fold discrepancy). The ratio of these activities for each fraction was then calculated. Fig 2.6. presents this ratio in graphical form. For the low mol wt ALP, the mean ratio was 69 (S.D. 7, N=15) whereas for high mol wt ALP, the mean ratio was 181 (S.D. 4, N=2), a discrepancy of nearly three-fold. This confirms that high mol wt ALP differed from liver ALP in its relative affinities for p-nitrophenyl phosphate and α-naphthyl phosphate and that this difference largely accounted for the observed discrepancies between the estimates of high mol wt ALP based on Sepharose 6B chromatography and polyacrylamide gel electrophoresis. Further investigations into this phenomenon are presented in section 3.2.4.

The results of the electrophoretic assay in this experiment are considerably lower than those obtained in Table 2.1. This difference can only be accounted for by small alterations in staining procedure which occurred during the intervening time, e.g. time allowed for colour development, temperature, activity of samples etc. The electrophoretic assay was sensitive to such small deviations from the standard method, a factor which led to distrust of its reliability as a quantitative assay.

Figure 2.6. Ratio of ALP activity using p-nitrophenyl phosphate as substrate to activity using α-naphthyl acid phosphate as substrate (both activities expressed in instrumental units). The ratio is plotted against the fractions obtained during Sepharose 6B chromatography of a serum containing liver and high mol wt ALP.



2.9. QUANTITATION OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE BY ION-EXCHANGE CHROMATOGRAPHY

Because it was tedious and time-consuming to use Sepharose 6B chromatography for the measurement of high mol wt ALP in large numbers of samples, an alternative method was developed based on ion-exchange chromatography. The method was suitable for the analysis of small batches of samples.

2.9.1. Ion-exchange method

Apparatus. Small columns were employed using 20 ml disposable plastic syringe barrels (internal diameter 2 cm). The gel matrix was supported by small circles of gauze. The upper seal of each column was formed by the original rubber syringe plunger end, through which was passed a small-diameter Teflon tube connected to a peristaltic pump.

Reagents. 1. DEAE-cellulose pre-cycled and equilibrated with buffer A.

2. Buffer A: 0.1 mol/l NaCl in 0.01 mol/l Tris-HCl buffer pH 7.5.

3. Buffer B: 0.3 mol/l NaCl in 0.01 mol/l Tris-HCl buffer pH 7.5.

Assay protocol. 20 ml DEAE-cellulose was poured into each column and washed through with Buffer A for two hours at a flow rate of 78 ml/hour in order to achieve complete

equilibration. The final bed volume was approximately 10 ml.

After equilibration the supernatant was removed and replaced with 1 ml serum which had been dialysed overnight against Buffer A. The serum was allowed to soak into the resin and was replaced with approximately 4 ml Buffer A. Buffer A (30 ml) was then pumped through the column, and a single 30 ml fraction collected, designated Fraction I.

Pumping was stopped, the supernatant removed and replaced with approximately 4 ml Buffer B. A second elution using Buffer B was carried out, a single 30 ml fraction again being collected, designated Fraction II (this fraction contained high mol wt ALP).

The ALP activities of the undialysed serum, Fraction I and Fraction II were measured. The activity of high mol wt ALP was calculated from the formula:

$$\text{High mol wt ALP activity} = \frac{(\text{activity Fraction II}) \times (\text{activity of serum}) \text{ iu/l}}{(\text{activity Fraction I} + \text{activity Fraction II})}$$

It was possible to analyse up to 8 sera in each batch. The whole assay, including measurement of activities and calculation of results, takes about 2 hours to perform for each batch.

2.9.2. Validation of Conditions

Effect of ionic strength. 5 ml serum containing liver and

high mol wt ALP was applied to a 16 x 2.5 cm DEAE-cellulose column and eluted with a gradient of increasing ionic strength, using NaCl in 0.01 mol/l Tris-HCl pH 7.5. The elution profile showed two main peaks of activity (Fig 2.7.). The fractions corresponding to each of these peaks were pooled, concentrated and applied to a Sepharose 6B column. They were also subjected to electrophoresis on 7% polyacrylamide gel together with a serum marker containing liver and high mol wt ALP. By these means, it was shown that the second ion-exchange peak corresponded to the high mol wt ALP. The ionic-strength corresponding to the trough between the two peaks was 0.1 mol/l (the ionic strength of Buffer A).

Volume of fractions. The two-step ion-exchange method was carried out on a serum containing liver and high mol wt ALP except that, instead of collecting a single 30 ml fraction at each ionic strength, 20 x 2.5 ml fractions were collected at each ionic strength. ALP activity was measured in each of these fractions and the elution profile shown in Fig 2.8. was obtained. It can be seen that elution of the activity corresponding to each ionic strength was complete when 30 ml had been collected. This volume was therefore chosen for Fractions I and II.

Figure 2.7. Elution pattern of a serum containing liver and high mol wt ALP obtained by DEAE-cellulose chromatography on a 16 x 2.5 cm column.

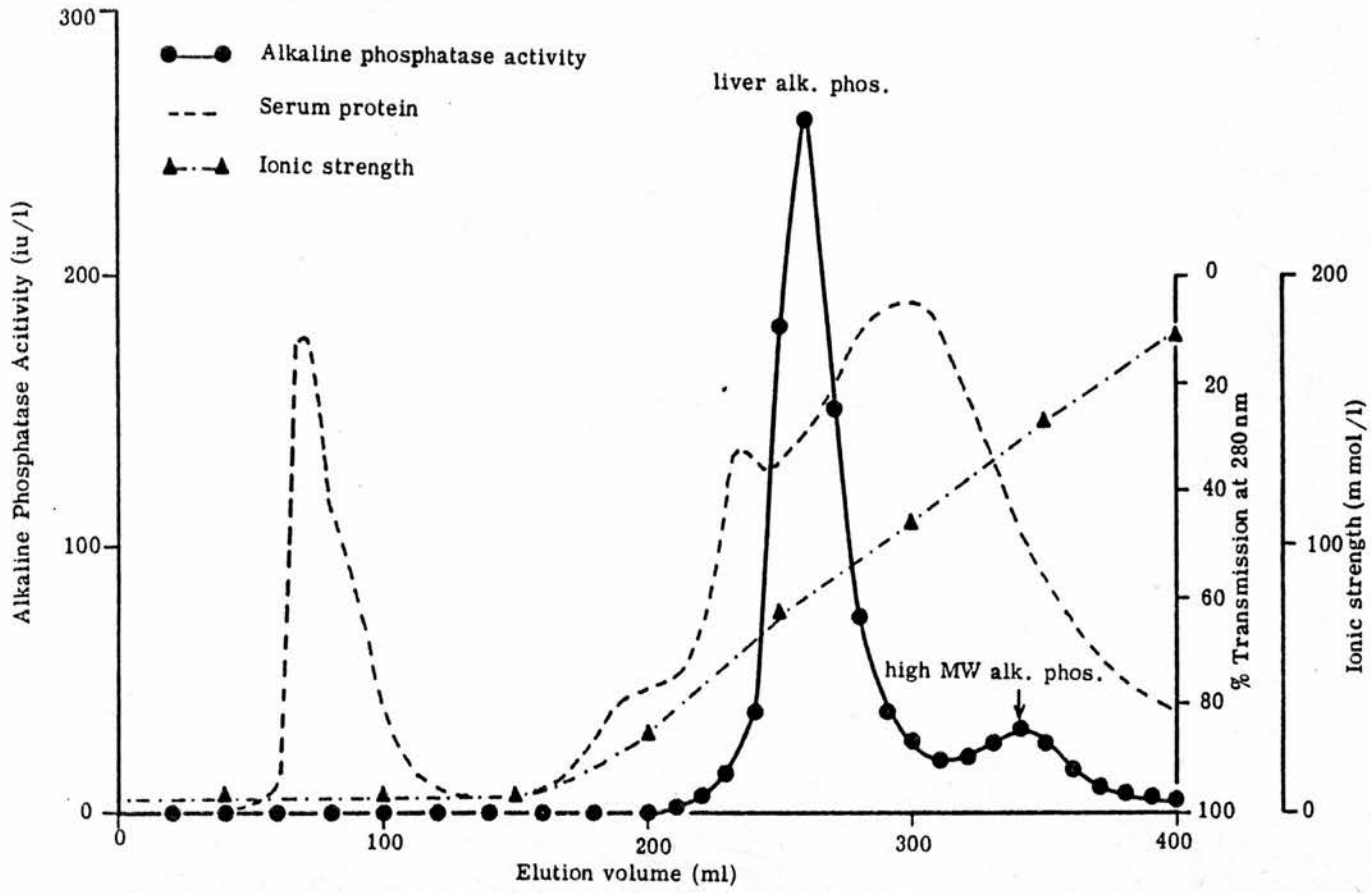
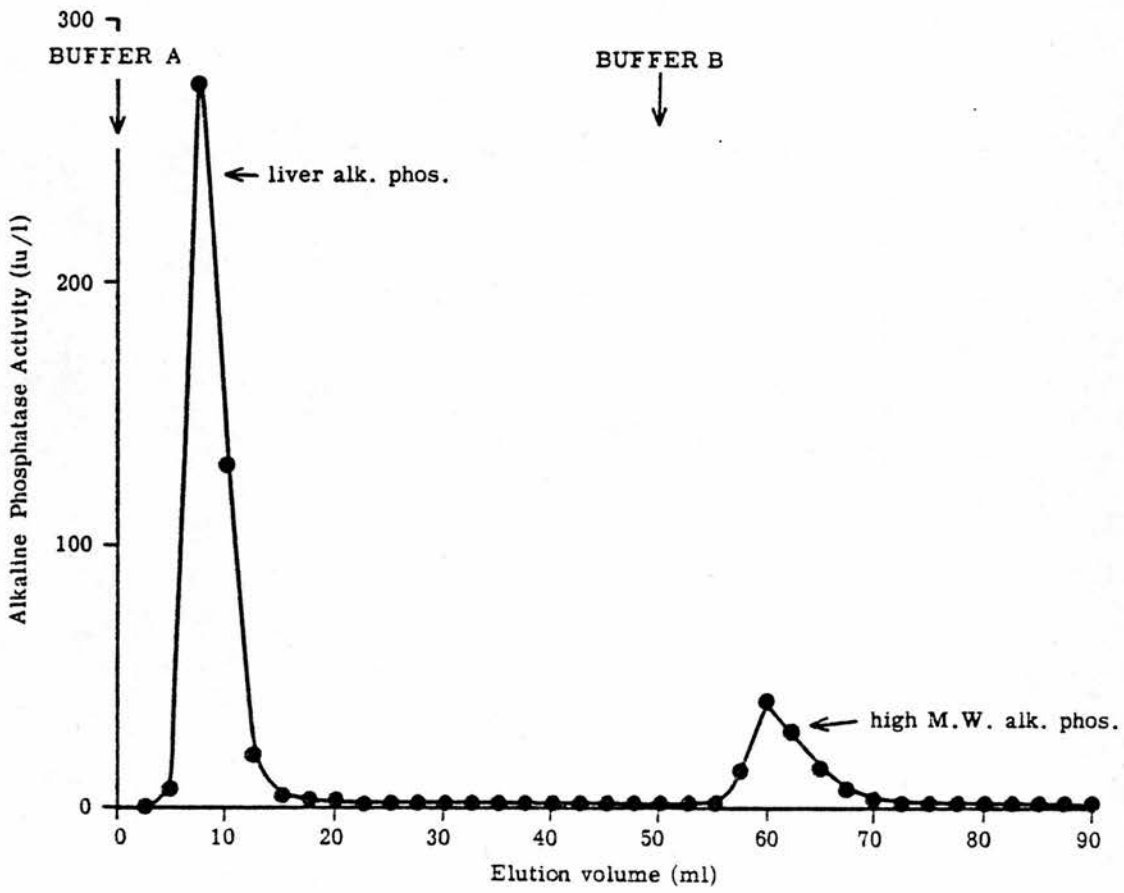


Figure 2.8. Elution pattern of a serum containing liver and high mol wt ALP obtained by the two step ion-exchange method. See text for details.

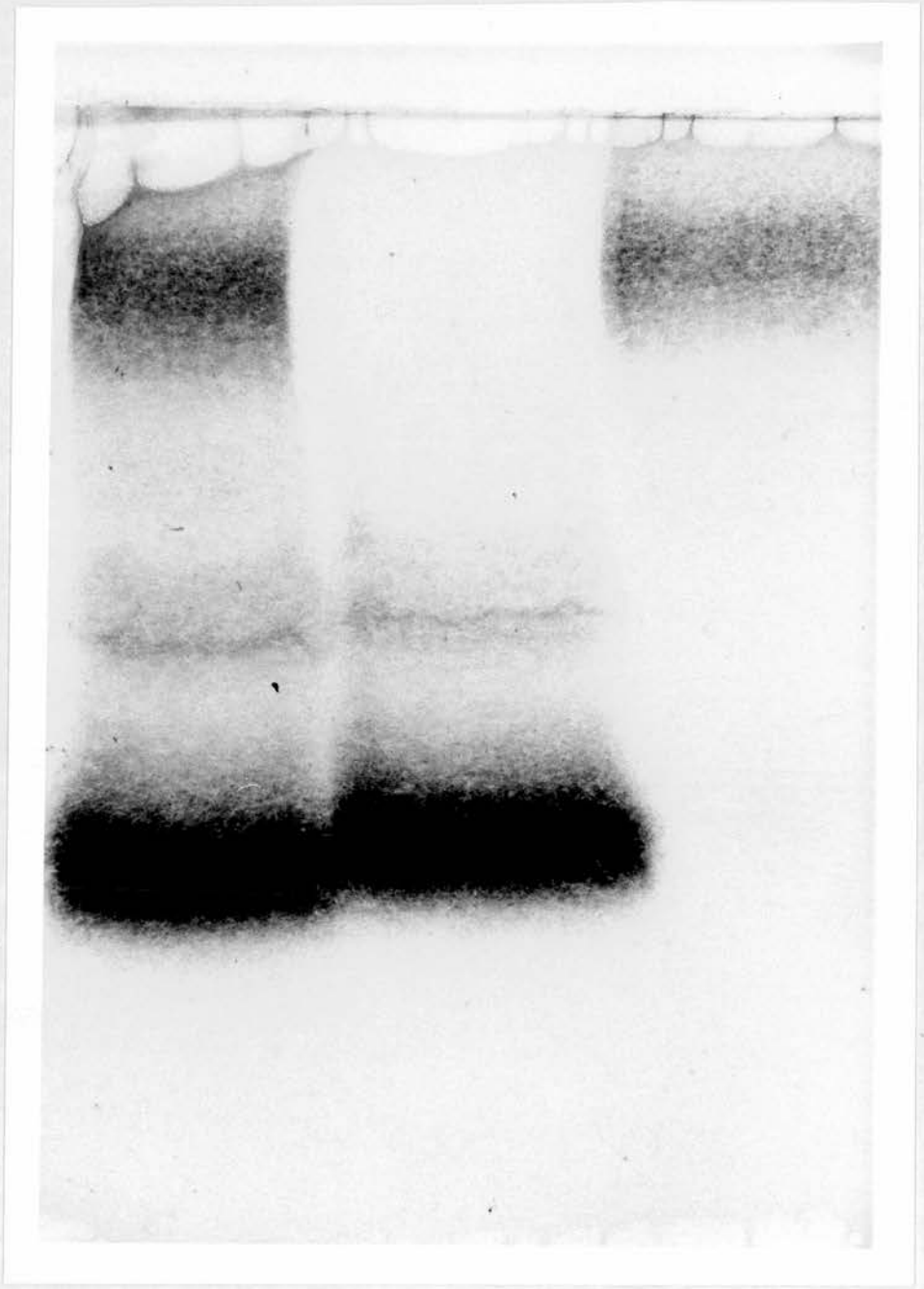


Identification of Fractions I and II; assessment of cross-contamination. A serum containing liver and high mol wt ALP only was analysed by the two-step ion exchange method. Fractions I and II were each concentrated and subjected to electrophoresis on 2.5% polyacrylamide gel. The undialysed serum was also subjected to electrophoresis as a marker. Fig 2.9. shows that Fraction I corresponded to the liver isoenzyme and Fraction II to high mol wt ALP. There was no detectable cross-contamination or carry-over.

Investigation of interference by other ALP isoenzymes. When the two-step ion-exchange method was carried out on 51 sera shown by electrophoresis to contain only liver and high mol wt ALP in varying proportions, the percentage activity found in Fraction II ranged from 4 to 35% (6 to 318 iu/l). In 24 sera, shown by electrophoresis to contain undetectable levels of high mol wt ALP but varying proportions of liver, bone, intestinal and placental isoenzymes, the percentage activity found in Fraction II ranged from 1 to 4% (1 to 9 iu/l).

One serum contained the rare slow band ALP (see Chapter 6) and gave 4% activity (7 iu/l) in Fraction II. Another serum contained the Regan variant (Crofton and Smith, 1978), a rare cancer-associated isoenzyme, in addition to the liver isoenzyme; this serum gave 9%

Figure 2.9. ALP electrophoresis patterns on 2.5% polyacrylamide gel. From left to right: a) serum containing liver and high mol wt ALP; b) Fraction I (concentrated) from this serum; c) Fraction II (concentrated) from this serum. O, origin; L, liver isoenzyme; H, high mol wt ALP. See text for details.



(16 iu/l) activity in Fraction II. Fractions I and II from the serum were each concentrated and subjected to electrophoresis on 2.5% polyacrylamide gel. Fraction II contained only the Regan variant and no high mol wt component was present.

Comparison with Sepharose 6B chromatography. Six sera with varying proportions of high mol wt ALP were analysed by the two-step ion-exchange method and by Sepharose 6B chromatography. ALP activities were measured in the fractions corresponding to the liver and high mol wt ALP peaks respectively. Correspondence between the two methods was good (Fig 2.10). Although the ion-exchange method appeared to give a slightly higher estimate of high mol wt ALP this was probably within the limits of analytical error for the two methods.

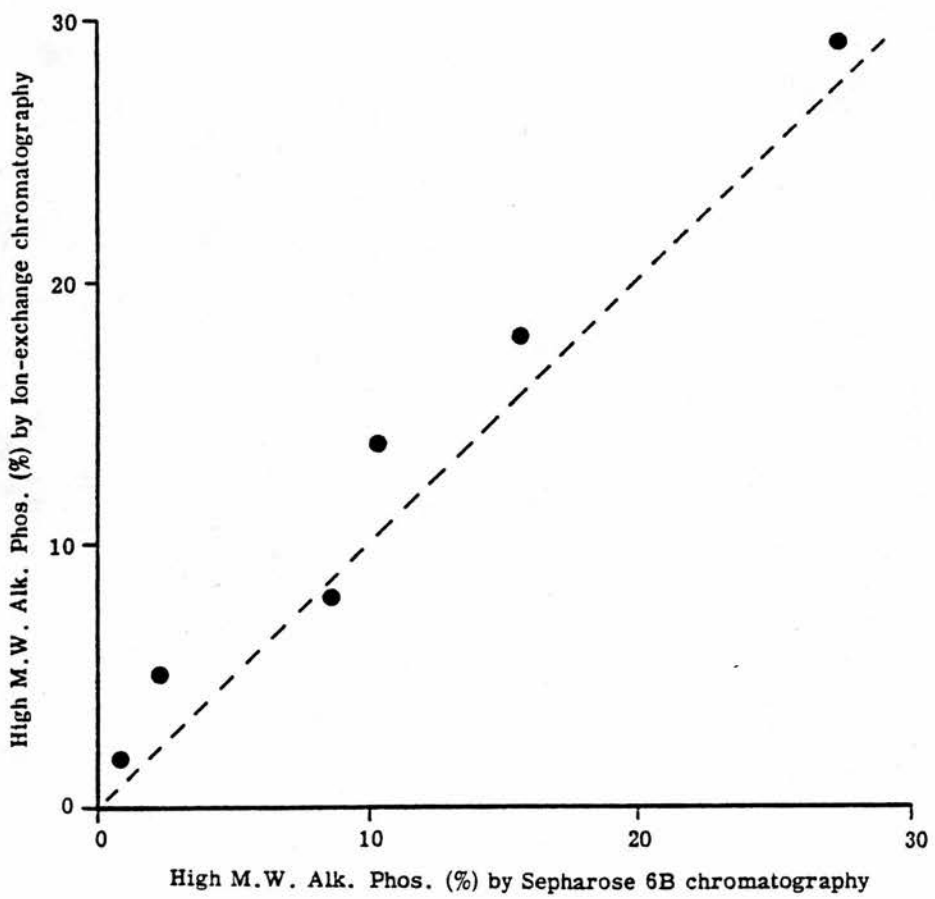
Precision and yield. Yields from the ion-exchange columns ranged from 85% to 105% with a mean of 95% (N=11).

The between-batch coefficient of variation (including the dialysis step) for high mol wt ALP measured by the two-step ion-exchange method was 3.4% (N=30, mean activity 102 iu/l, range 6 to 350 iu/l).

2.10. CHOICE OF METHODS FOR THE DETECTION AND MEASUREMENT OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE

The major characteristics by which high mol wt ALP

Figure 2.10. Comparison between high mol wt ALP activities measured by Sepharose 6B chromatography (X-axis) and ion-exchange chromatography (Y-axis) in 6 subjects. ---- 45° line.



differs from most of the other forms of ALP are its high mol wt (by definition) and its charge. Measurement by Sepharose 6B chromatography is based on the first of these characteristics, ion-exchange chromatography on the second and electrophoresis on both, depending on the medium used.

Molecular sieving column chromatography has been used most commonly in the past (Fennelly et al, 1969) in the detection and measurement of high mol wt ALP. However, a cautionary note should be struck in the comparison of Sephadex G200 chromatography with Sepharose 6B, 4B and 2B chromatography as methods of measuring high mol wt ALP. Sephadex G200 has a smaller mesh size than the Sepharose range of gels and its void volume peak therefore contains proteins of lower mol wt e.g. α_2 macroglobulin, IgM and most of the α and β lipoproteins. This is reflected in the protein elution pattern which shows a detectable void volume peak in Sephadex but not in Sepharose. Therefore the two types of gel may not be measuring the same thing. For instance, Jennings et al (1970) found that the void volume peak from a Sephadex G200 column contained two ALP components, one of which remained at the origin during starch gel electrophoresis and one of which migrated in the β lipoprotein position. If only the higher mol wt of these two ALP components, namely that which remains at the origin,

is regarded as the true 'high mol wt ALP' specific for liver disease, then Sephadex G200 chromatography overestimates it. Only Sepharose gels which do not exclude such proteins as β lipoprotein and have little or no detectable protein in their void volumes will provide an accurate estimate of the high mol wt ALP which is specific for liver disease and with which this thesis is concerned. Sepharose gel chromatography must therefore be regarded as the reference method, although it is slow and tedious.

The technique of quantitation by densitometry of cellulose acetate strips following electrophoresis and staining by gel overlay was first described by Rhone et al (1973). I have found this method to be very imprecise, presumably owing to diffusion and variable background staining. In addition, the agar overlay technique was inaccurate since it overestimated the amount of high mol wt ALP in serum. Fixative techniques have been advocated to prevent diffusion before staining (Siede and Seiffert, 1977), thereby improving precision, but these are tedious. Moreover, since the fixative bath contains ethanol and lead acetate, the enzyme may be adversely affected.

Of the electrophoretic media investigated, 2.5% polyacrylamide gel appeared to give the most reproducible results and showed an acceptable linearity between colour

intensity and activity. No other isoenzyme interfered with the measurement. There was poor correlation between results obtained using 2.5% polyacrylamide gel and those using alternative media, but this could be explained by the poor precision which was generally found. The major disadvantages of this apparently simple method of measuring high mol wt ALP were its lack of long-term reliability and the lack of concordance between the results obtained by electrophoresis and those obtained by Sepharose 6B chromatography. This phenomenon was largely attributable to an apparent difference in substrate specificities between high mol wt ALP and the liver isoenzyme at the substrate concentrations employed (see section 3.2.4.). It may also have been partly attributable to the use of a diazo dye which inhibits the enzyme reaction to some extent, making linear development of colour difficult to achieve (Rhone and Mizuno, 1972; Fritsche and Adams-Park, 1972). For this reason electrophoresis on 2.5% polyacrylamide gel was abandoned as a quantitative technique but retained as a qualitative tool to demonstrate the presence or absence of high mol wt ALP.

Another electrophoretic method for measuring high mol wt ALP, based on elution from sequential strips of polyacrylamide gel followed by measurement of activities

in each eluate, has been described by Price and Sammons (1974). This is a time-consuming procedure. Furthermore, recovery of enzyme activity from macerated gel may vary from isoenzyme to isoenzyme, from serum to serum and from gel to gel. In particular, it seems likely that large molecules will be more difficult to recover from the gel than small molecules and that the proportion may be critically dependent on the characteristics of the gel, such as its strength and degree of cross-polymerisation, as well as the conditions of elution.

The ion-exchange method was found to be relatively rapid, reliable and precise. The common isoenzymes of ALP, namely those of liver, bone, intestinal and placental origin, and the slow band ALP (see Chapter 6) which is occasionally encountered, did not appear to interfere. Only the Regan variant (Crofton and Smith, 1978), as might be expected from its high electrophoretic mobility and presumably greater charge, eluted in Fraction II. This is a rare cancer-associated isoenzyme, much less commonly encountered than the better-known Regan isoenzyme, and the proportion of cases in which its presence would interfere with the ion-exchange method is likely to be very small.

There was fairly good correlation between estimates of high mol wt ALP obtained by the ion-exchange method

and by Sepharose 6B chromatography, taking into account the fact that the two methods are based on different properties of high mol wt ALP. Although the slightly higher results obtained by the ion-exchange method could be due to a small amount of carry-over, the evidence fails to support this. It is probable that the slight discrepancy between the two methods was within the limits of their analytical error.

In summary, Sepharose 6B was chosen as the reference method for measuring high mol wt ALP in serum, but for routine measurements the two-step ion-exchange method was used. 2.5% polyacrylamide gel electrophoresis was employed for the qualitative demonstration of high mol wt ALP.

CHAPTER 3

PURIFICATION AND KINETICS

3.1. PURIFICATION OF ALKALINE PHOSPHATASE ISOENZYMES FROM SERUM AND BILE

All isoenzymes were purified from serum or bile rather than from tissue sources because high mol wt ALP in serum or bile may not necessarily occur in the same form in the tissues. Also, tissue extraction generally requires solubilisation with butanol (Morton, 1954) which destroys high mol wt ALP (section 4.7.1.). Since the activity of ALP in serum and bile is small relative to activities of liver or intestinal homogenates, and since large quantities of serum were not available it was not possible to achieve high degrees of purity in the preparations, nor was this attempted. The aim instead was a degree of purification sufficient to separate the isoenzymes from one another and from most of the contaminating plasma proteins. This would enable valid comparisons to be made between the isoenzymes but must be viewed against a purity recently attained for the liver isoenzyme of 1450 units/mg specific activity (Latner and Hodson, 1976).

3.1.1. Preparation of serum and bile

Serum containing either liver and high mol wt ALP or bone ALP was dialysed overnight at 4^{o}C against 0.01 mol/l

Tris-HCl buffer pH 7.5. The serum was then centrifuged to remove any precipitate and the supernatant was designated Fraction I.

Normal hepatic bile was collected via a T-tube from patients following gallbladder removal. The bile was mixed with an equal volume of 10 g/l protamine sulphate in water in order to precipitate lipid and pigment (Price et al, 1972). After centrifugation, the supernatant, which was a clear pale yellow colour, was concentrated 10-fold by negative pressure ultra-filtration and dialysed overnight at 4°C against 0.01 mol/l Tris-HCl buffer pH 7.5. The dialysed bile was also designated Fraction I.

3.1.2. Ion-exchange chromatography

5 ml Fraction I was applied to a DEAE cellulose column (approximately 16 x 2.5 cm) pre-equilibrated with 0.01 mol/l Tris-HCl buffer pH 7.5. The column was washed with one bed volume of this starting buffer. A salt gradient was then set up with 500 ml starting buffer in the mixing chamber and 500 ml 1 mol/l NaCl in 0.01 mol/l Tris-HCl buffer pH 7.5 in the reservoir. The flow rate was 120 ml/hour. The protein content of the eluate was monitored at 280 nm and 5 ml fractions were collected. ALP activities and the ionic strengths in the fractions

were measured. The overall yield of ALP from the column was approximately 90%.

Up to two peaks of ALP activity were eluted from serum and bile (Fig 3.1). In serum, there were three protein peaks whereas bile contained only one broad protein peak. The fractions corresponding to the peaks of ALP activity were pooled and concentrated approximately 10-fold by negative pressure ultrafiltration. The concentrates were designated Fractions IIA (for the first peak of activity eluting at an ionic strength of 80 mmol/l) and IIB (for the second peak of activity eluting at an ionic strength of 130 mmol/l) respectively.

The peaks eluted from the ion-exchange column were identified by their position during subsequent elution from the Sepharose 6B column (Fig 3.2, section 3.1.3.). They were also identified by electrophoresis on 2.5% polyacrylamide gel.

The mean ionic strengths at which various isoenzymes eluted were as follows:

liver isoenzyme: 80 mmol/l (range 72-86 mmol/l)
bone isoenzyme: 81 mmol/l (range 77-85 mmol/l)
low mol wt biliary component: 75 mmol/l
high mol wt serum component: 135 mmol/l (range 130-140 mmol/l)
high mol wt biliary component: 120 mmol/l

The liver and bone isoenzymes in serum and the low mol wt component(s) in bile therefore eluted in similar positions

Figure 3.1. Purification Stage 2. Elution patterns of ALP in serum and bile, obtained by DEAE-cellulose chromatography on a 16 x 2.5 cm column. —●— ALP activity; - - - protein elution profile; —·▲—· ionic strength gradient.

(a) serum containing liver and high mol wt ALP only.

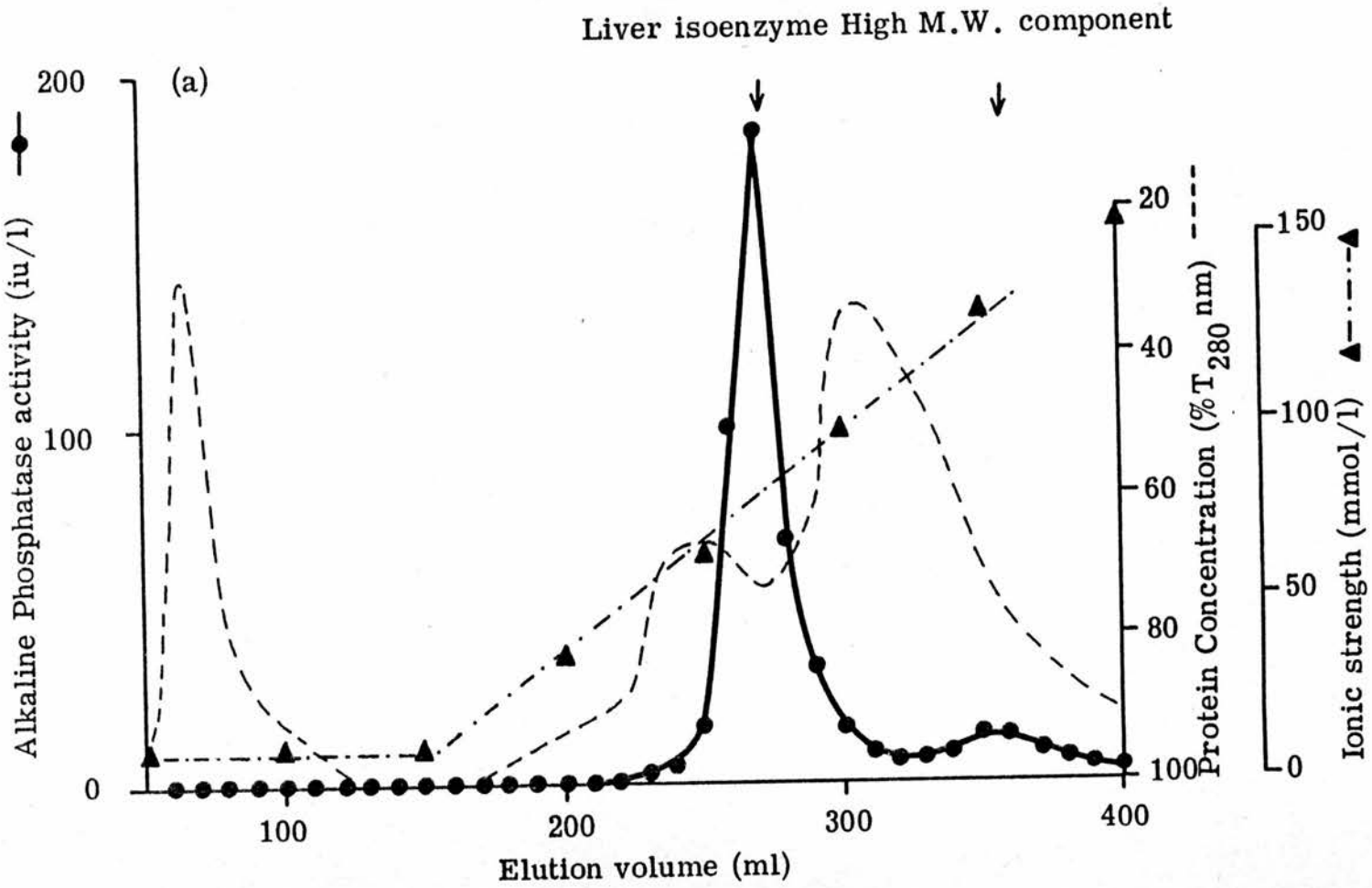


Figure 3.1. (continued).

(b) serum containing largely bone ALP

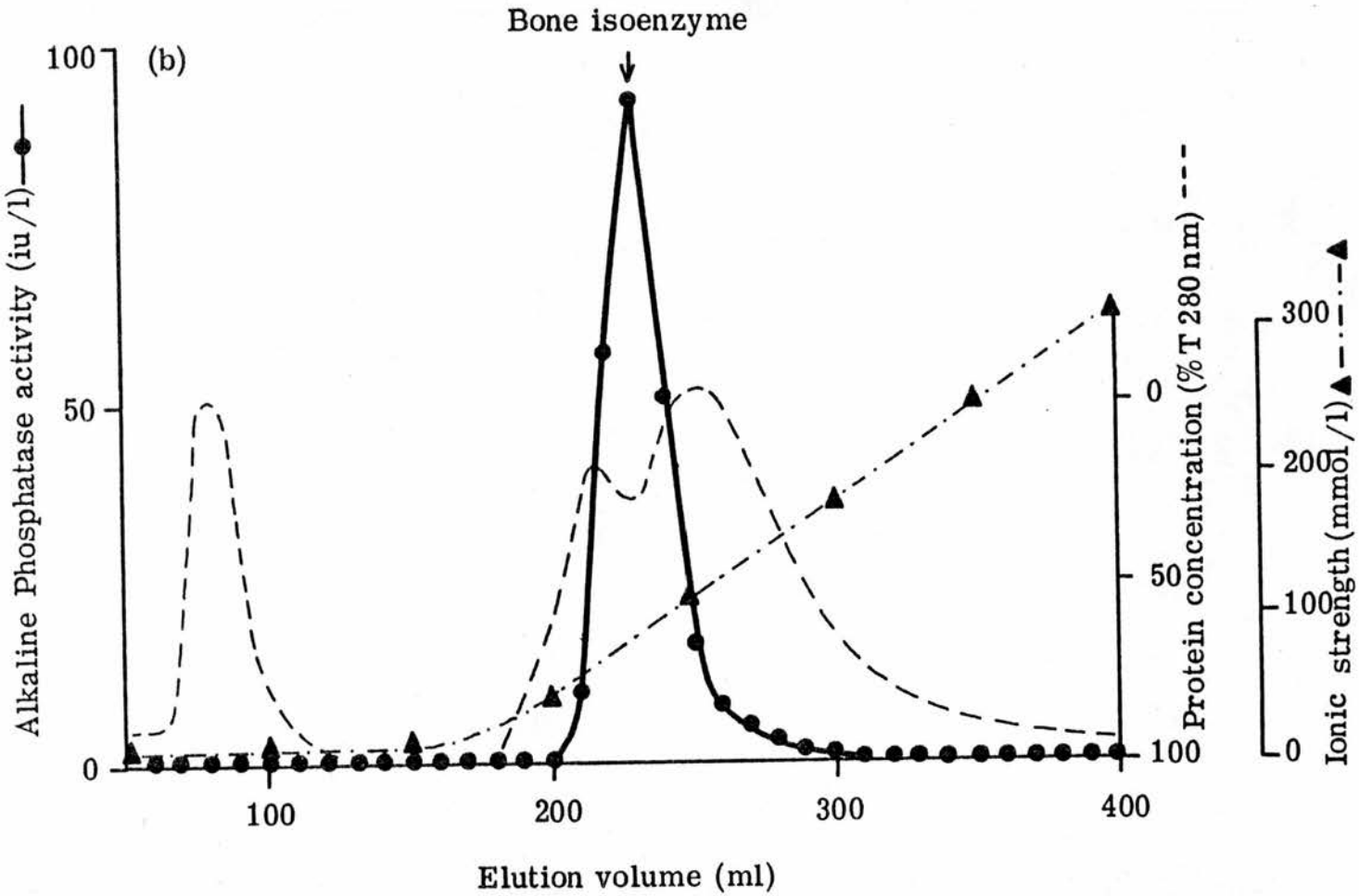
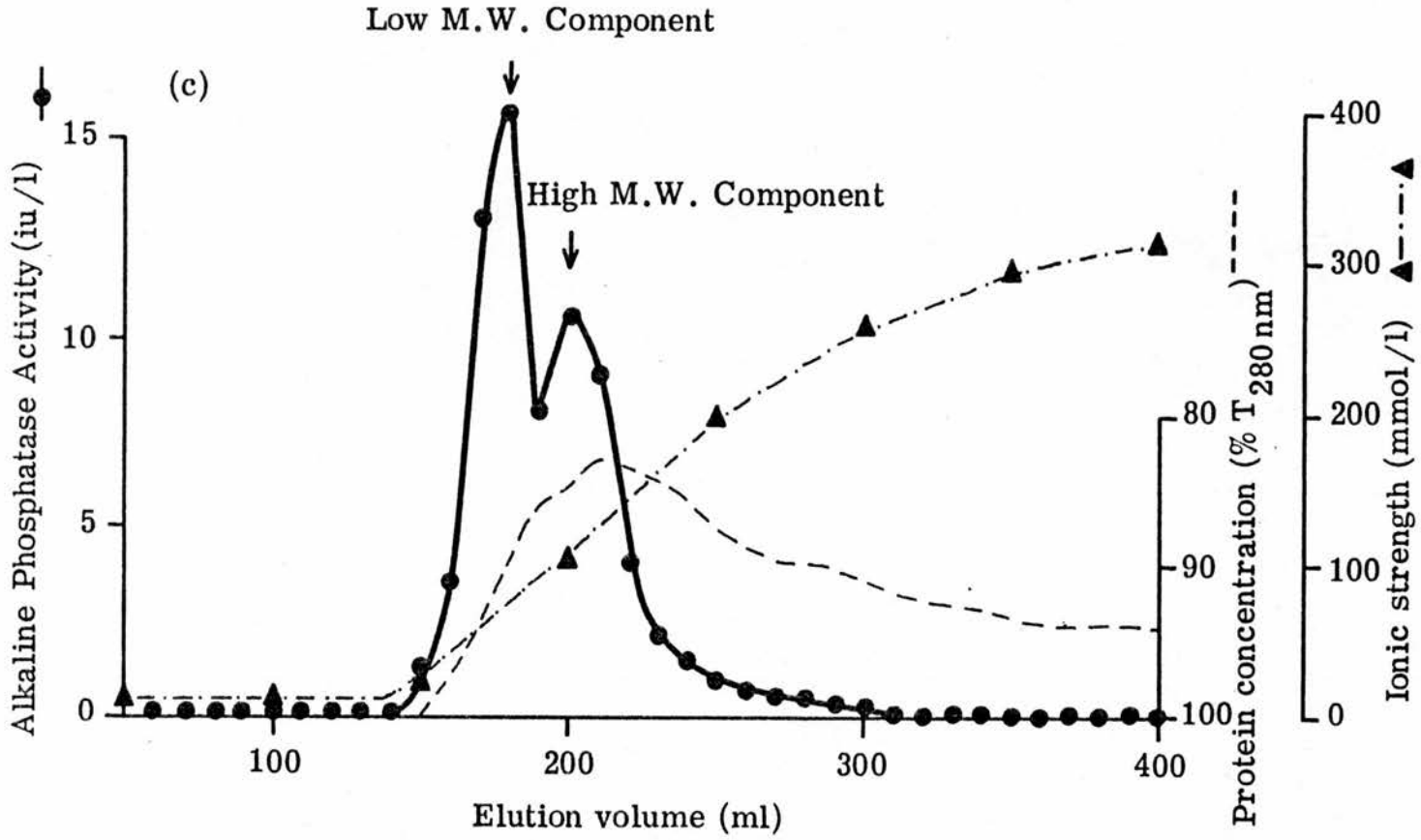


Figure 3.1. (continued).

(c) hepatic bile containing low and high mol wt ALP



(Fraction IIA). The high mol wt components in serum and bile also eluted in similar positions (Fraction IIB).

3.1.3. Sepharose 6B chromatography

2 ml of Fractions IIA and IIB were separately applied to a 90 x 2.5 cm Sepharose 6B column pre-equilibrated with 0.1 mol/l Tris-HCl buffer pH 7.7 containing 0.05 mol/l NaCl. Elution was carried out overnight at a flow rate of 28 ml/hour. The protein content of the eluate was monitored at 280 nm and 5 ml fractions were collected. The ALP activities in these fractions were measured.

Fraction IIA contained only liver or bone ALP from serum, or the low mol wt component(s) from bile, with no high mol wt ALP present (Fig 3.2.(a)). Two protein peaks were present in Fraction IIA derived from serum, but protein was undetectable in Fraction IIA derived from bile. The fractions corresponding to the ALP peak were pooled and concentrated 15 to 20-fold by negative pressure ultra-filtration. The concentrate was designated Fraction IIIA and represented partial purifications of liver or bone ALP from serum or low mol wt ALP from bile.

Two protein peaks were also present in Fraction IIB derived from serum but again protein was undetectable in Fraction IIB derived from bile (Figs 3.2.(b) and 3.2.(c)).

Figure 3.2. Purification Stage 3. Elution patterns of ALP in Purification Fraction II from serum and bile, obtained by Sepharose 6B chromatography on a 90 x 2.5 cm column. —●— ALP activity from serum; —▲— ALP activity from bile; - - - protein elution profile (Fraction IIA from serum only).

(a) Purification Fraction IIA (see text) from serum and bile

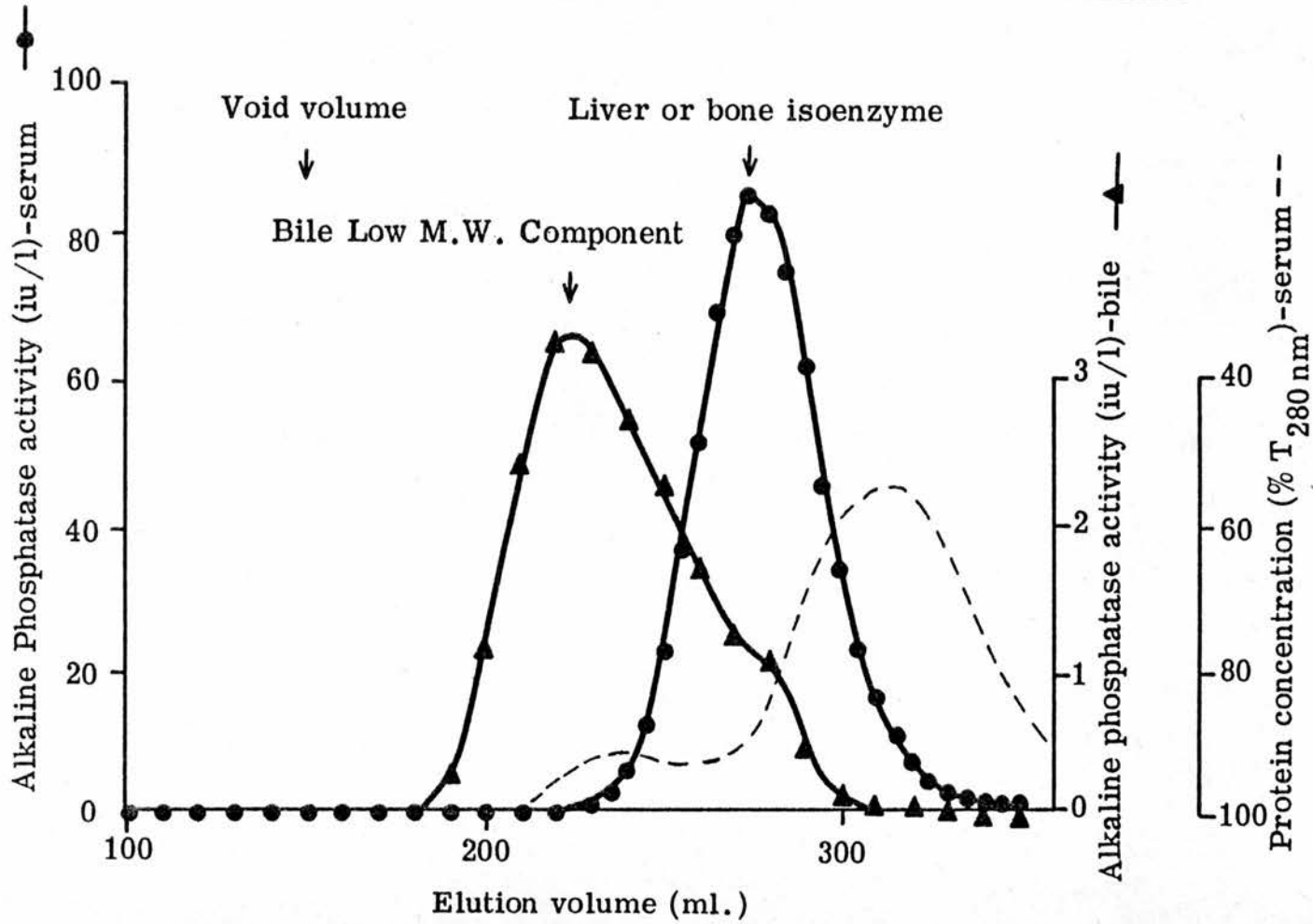


Figure 3.2. (continued)

(b) Purification Fraction IIB (see text) from serum

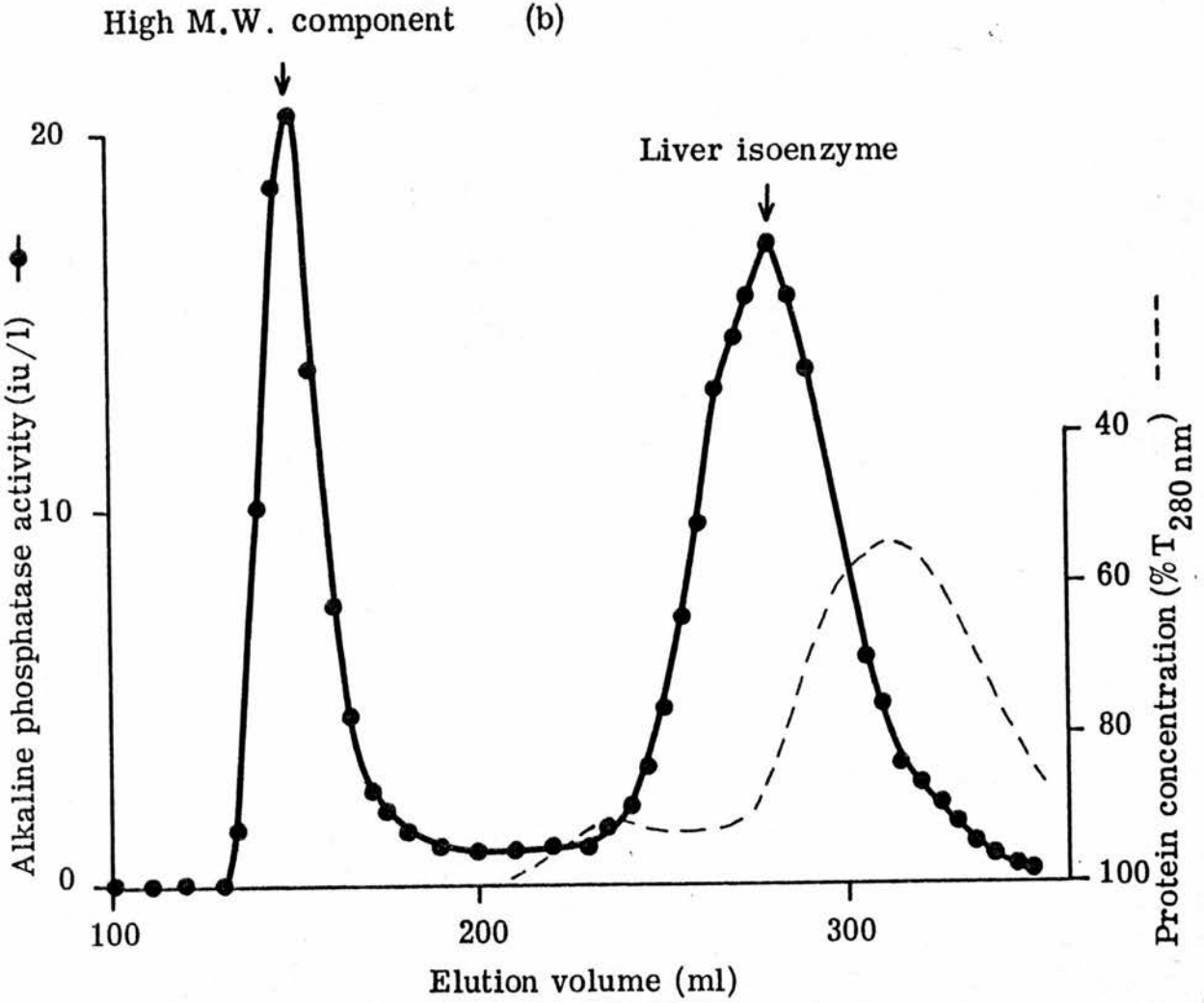
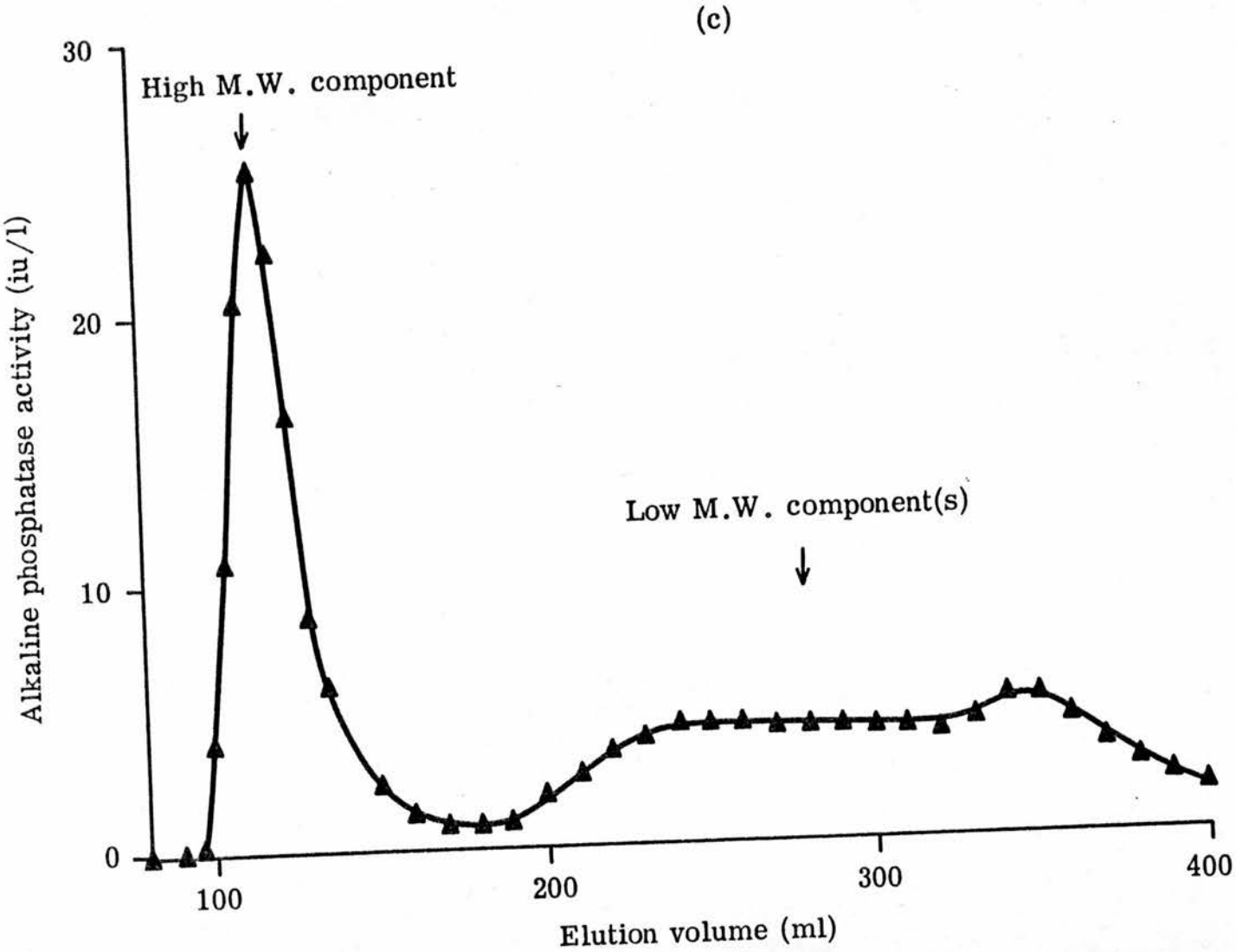


Figure 3.2. (continued).

(c) Purification Fraction IIB (see text) from bile



Fraction IIB contained a mixture of high and low mol wt ALP owing to incomplete resolution of the peaks on ion-exchange chromatography. However, only the fractions corresponding to the high mol wt peak were pooled and concentrated to give Fraction IIIB. This represented a partial purification of the high mol wt components from serum and bile. All partially purified preparations were stored at -20°C.

It was found that for high mol wt ALP from serum and bile higher yields with similar degrees of purification could be attained by omission of the ion-exchange stage in purification. This stage was therefore left out in routine purification of the high mol wt components from serum and bile.

The ALP activities of Fractions I to III were measured and their protein content estimated by the Lowry method (Lowry et al, 1951).

Typical purification schedules for liver, bone and high mol wt ALP from serum are shown in Table 3.1. For the liver isoenzyme, the degree of purification ranged from 27- to 48-fold. For the high mol wt component the degree of purification ranged from 189- to 236-fold for the three-stage purification scheme, but actually improved to 326-fold (with a 60% yield) for the two-stage scheme. This was presumably because in the ion-exchange elution

TABLE 3.1.

Purification schedule for ALP isoenzymes from serum

Isoenzyme	Purification fraction	Activity (iu/l)	Protein (g/l)	Specific activity (iu/g)	Purification (-fold)	Yield (%)
Liver	I	1475	58.3	25.3	1	100
	IIA	1251	2.35	532	21	59
	IIIA	302	0.342	882	35	27
Bone	I	525	55.2	9.5	1	100
	IIA	480	7.40	64.9	7	53
	IIIA	162	0.757	214	23	20
High mol wt	I	321*	50.0	6.4	1	100
	IIB	209	8.66	24.2	4	61
	IIIB	51	0.042	1217	189	20

* The activity of high mol wt ALP in the original serum was calculated from the proportions of liver and high mol wt ALP observed on ion-exchange chromatography of the serum.

pattern, the high mol wt component peak coincided with the main albumin peak.

Similar activities and yields were obtained for the partially purified preparations of low and high mol wt ALP from bile, but purification schedules cannot be given. This is because the protein concentration in Fraction I of bile could not be measured either by the Lowry method or by the Coomassie Brilliant Blue method (Bradford, 1976) owing to interference by some component in bile.

3.2. ENZYMIC PROPERTIES

Because high mol wt ALP rises in serum only in patients with liver disease, it was thought that it might be related to the liver isoenzyme as regards its structure and properties. To test this hypothesis, the enzymic properties of high mol wt ALP were compared principally with those of the liver isoenzyme. Since bile also contains a high mol wt ALP which may be related to the serum high mol wt ALP (similar mol wts and charges), this was included in many of the kinetic studies. The bone isoenzyme from serum and low mol wt ALP from bile were also investigated for comparison.

All studies on the enzymic properties were done on the partially purified preparations of ALP described in section 3.1.

3.2.1. Statistical analysis of experimental data

In the analysis of enzyme kinetics, the fitted lines in the Lineweaver Burk plots were based on estimates of K_m and V_{max} obtained by a least-squares non-linear fitting procedure of the direct velocity versus substrate concentration data points (Wilkinson, 1961). This procedure assumes equal variance of each of the measured data points. The fitted lines in the velocity^{-1} versus inhibitor concentration plots were obtained by a weighted regression procedure which assumed equal variance for each velocity and weighted the variance of each velocity^{-1} data point accordingly. The calculations and graphical output were programmed on a Hewlett-Packard 9821 Desk Calculator equipped with Printer/Plotter.

One way analysis of variance on the 5 estimates of K_i for each isoenzyme was carried out according to the fixed effects model in which K_i was regarded as a fixed but unknown quantity to be determined (Snedecor and Cochran , 1967). This analysis assumes that the data are normally distributed (although the analysis is insensitive to minor departures from normality) and that the variances in all classes are equal. The latter assumption was tested (Snedecor and Cochran, 1967) and found to be true. The Scheffé method (Snedecor and Cochran, 1967) was used

to test for differences between the mean K_is for each isoenzyme since this method is appropriate when the difference has not been anticipated by the experimenter.

3.2.2. Buffer type, concentration and pH optima

The activities of liver and high mol wt ALP were measured in various concentrations of three transphosphorylating buffers, namely:

1. Diethanolamine (DEA) buffer pH 10.2
2. 2-amino-2-methylpropan-1-ol (AMP) buffer pH 10.2
3. Tris-borate buffer pH 9.5.

All buffers contained 0.5 mmol/l $MgCl_2$ and the concentration of the substrate, p-nitrophenyl phosphate, was 14 mmol/l in each case.

DEA buffer was the most sensitive buffer for both isoenzymes and Tris-borate the least sensitive (Fig 3.3.). Both isoenzymes exhibited optimal activities at similar buffer concentrations, namely:

1. DEA buffer pH 10.2 : 1.5 mol/l
2. AMP buffer pH 10.2 : 0.5 mol/l
3. Tris-borate buffer pH 9.5 : 0.5 mol/l

The pH optima of liver and high mol wt ALP in the three buffers at these optimal buffer concentrations are shown in Fig 3.4. Again both isoenzymes showed similar pH optima, namely:

Figure 3.3. Effect of buffer type and concentration on ALP activities in partially purified ALP preparations. —▲— DEA -HCl pH 10.2; —●— AMP-HCl pH 10.2; —■— Tris-borate pH 9.5.

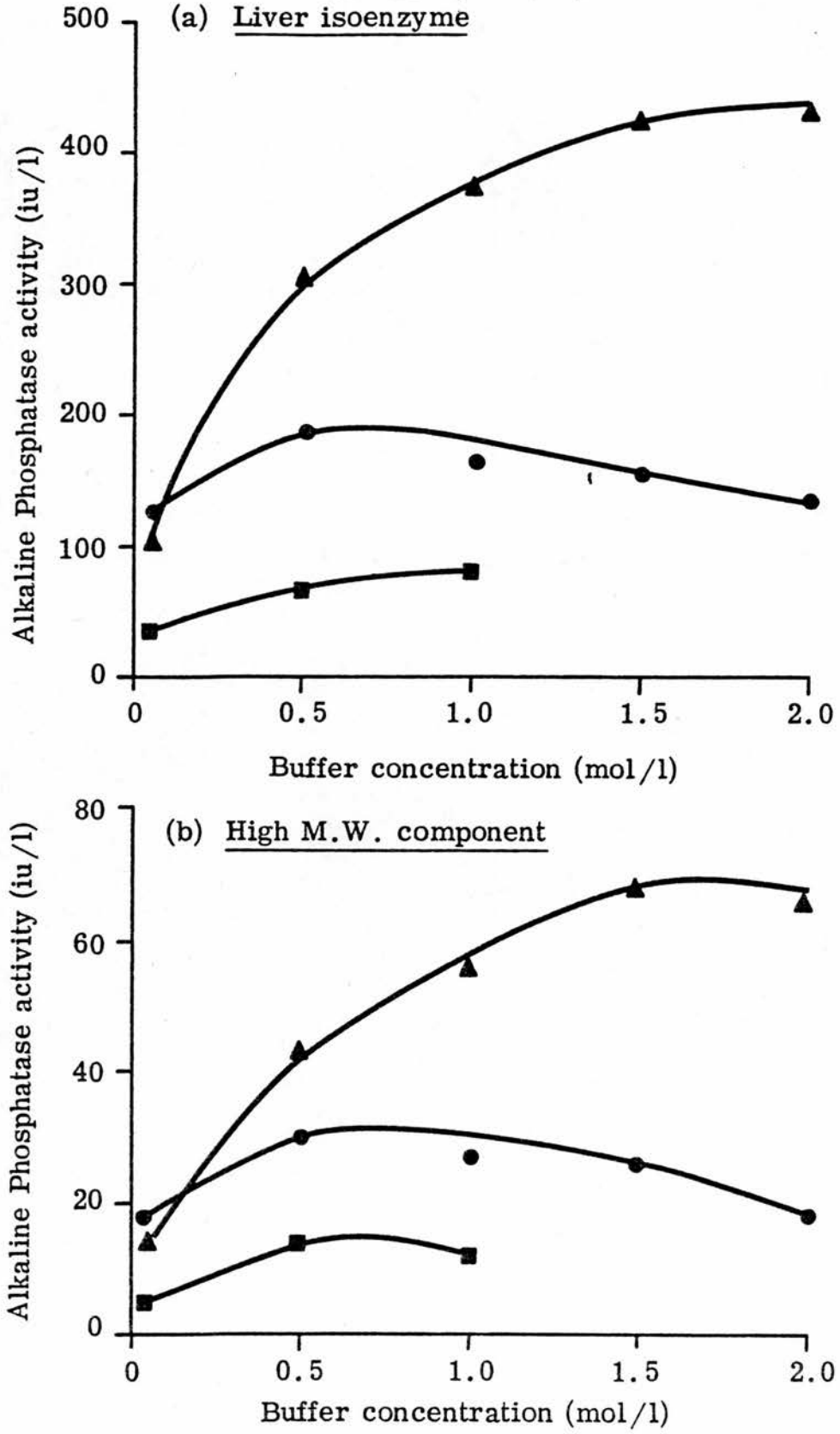
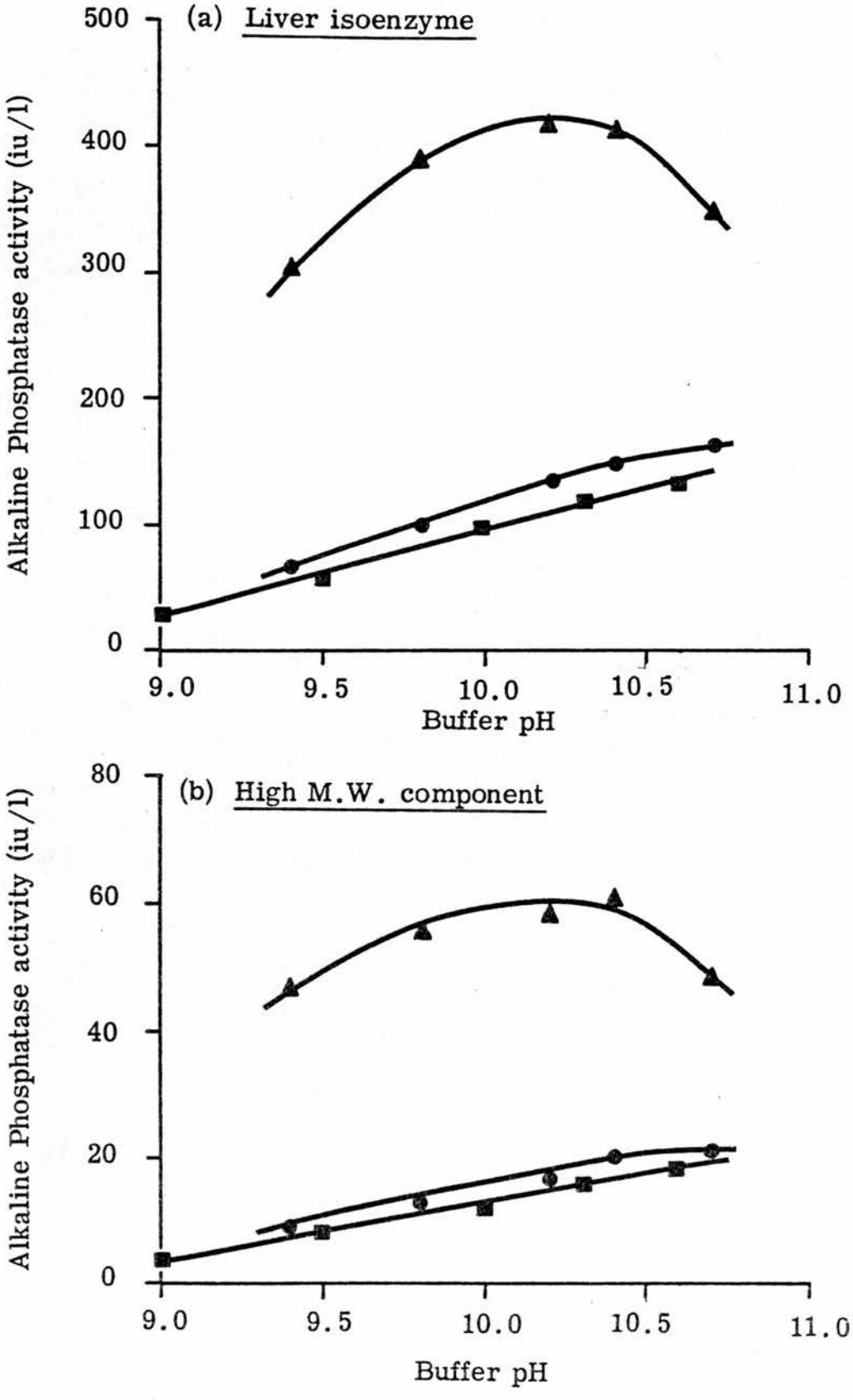


Figure 3.4. Effect of buffer type and pH on ALP activities in partially purified ALP preparations. —▲— 1.5 mol/l DEA-HCl; —●— 0.5 mol/l AMP-HCl; —■— 0.5 mol/l Tris-borate.



1. 1.5 mol/l DEA buffer : pH 10.2

2. 0.5 mol/l AMP buffer : pH 10.7

3. 0.5 mol/l Tris-borate buffer : pH 10.6

The buffer conditions found to be optimum for the assay of liver and high mol wt ALP agree well with those found previously for the liver isoenzyme (McComb and Bowers, 1972; Bretaudière et al, 1977).

Because DEA was the most sensitive transphosphorylating buffer and because its pH optimum with respect to ALP was better defined, it was used in subsequent experiments at a concentration of 1.5 mol/l and pH 10.2.

3.2.3. Linearity of velocity versus enzyme concentration

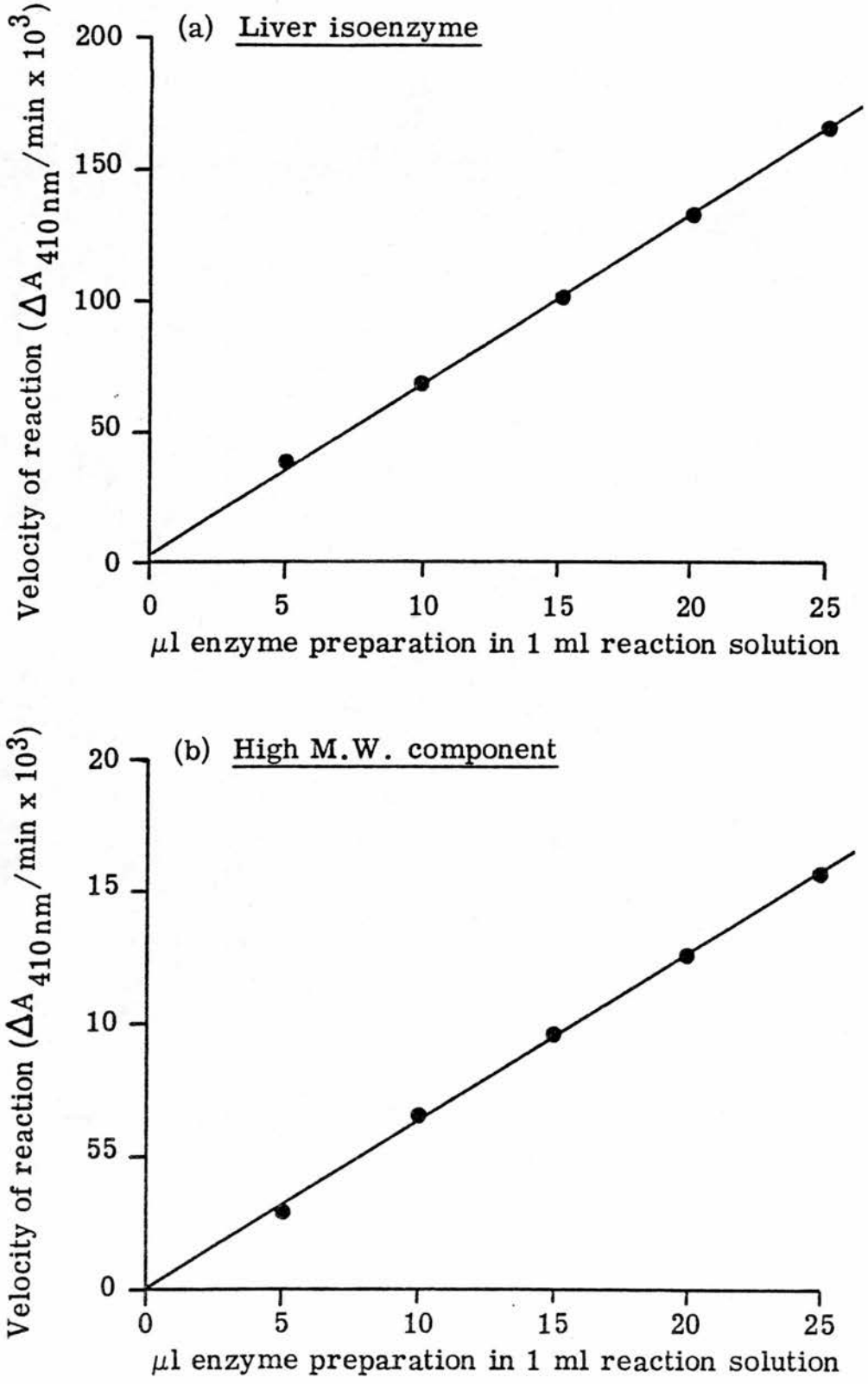
The activities of various dilutions of liver and high mol wt ALP were measured in 1.5 mol/l DEA-HCl buffer pH 10.2 containing 0.5 mmol/l $MgCl_2$ (Fig 3.5.). The substrate was 14 mmol/l p-nitrophenyl phosphate.

The graph of velocity versus enzyme concentration was linear for both isoenzymes.

3.2.4. K_ms

α-Naphthyl phosphate as substrate. In order to compare substrate specificities, the activities of the following ALP preparations were measured at various concentrations of the substrate α-naphthyl acid phosphate in 1.5 mol/l DEA-HCl buffer pH 10.2 containing 0.5 mmol/l $MgCl_2$:

Figure 3.5. Relationship of reaction velocity to enzyme concentration using partially purified ALP preparations.



a) liver ALP b) bone ALP c) high mol wt serum ALP d) high mol wt biliary ALP e) low mol wt biliary ALP.

Fig 3.6. shows the velocity^{-1} versus [α-naphthyl phosphate]$^{-1}$ Lineweaver-Burk plots for each isoenzyme investigated. The K_ms of the isoenzymes (Table 3.2.) were similar, though not identical, for all the ALP isoenzymes tested except high mol wt serum ALP which had a K_m approximately twice that of the other isoenzymes, a significant difference.

TABLE 3.2.

K_ms with respect to α-naphthyl acid phosphate as substrate.

ALP isoenzyme	Km (α naphthyl phosphate) (mmol/l)	S.E. Km estimate (single data set) (mmol/l)
Liver ALP	0.21	0.02
Bone ALP	0.15	0.01
High mol wt serum ALP	0.39	0.02
High mol wt biliary ALP	0.21	0.01
Low mol wt biliary ALP	0.17	0.01

p-Nitrophenyl phosphate as substrate. Similarly, the activities of the ALP isoenzyme preparations were measured at varying concentrations of p-nitrophenyl phosphate in the same buffer. The K_ms of the isoenzymes (Table 3.3.)

Figure 3.6. Lineweaver-Burk plots of 1/v versus [αnaphthyl acid phosphate]$^{-1}$ for each ALP preparation. V is measured in terms of absorbance change per min at 340 nm.

(a) Liver alkaline phosphatase

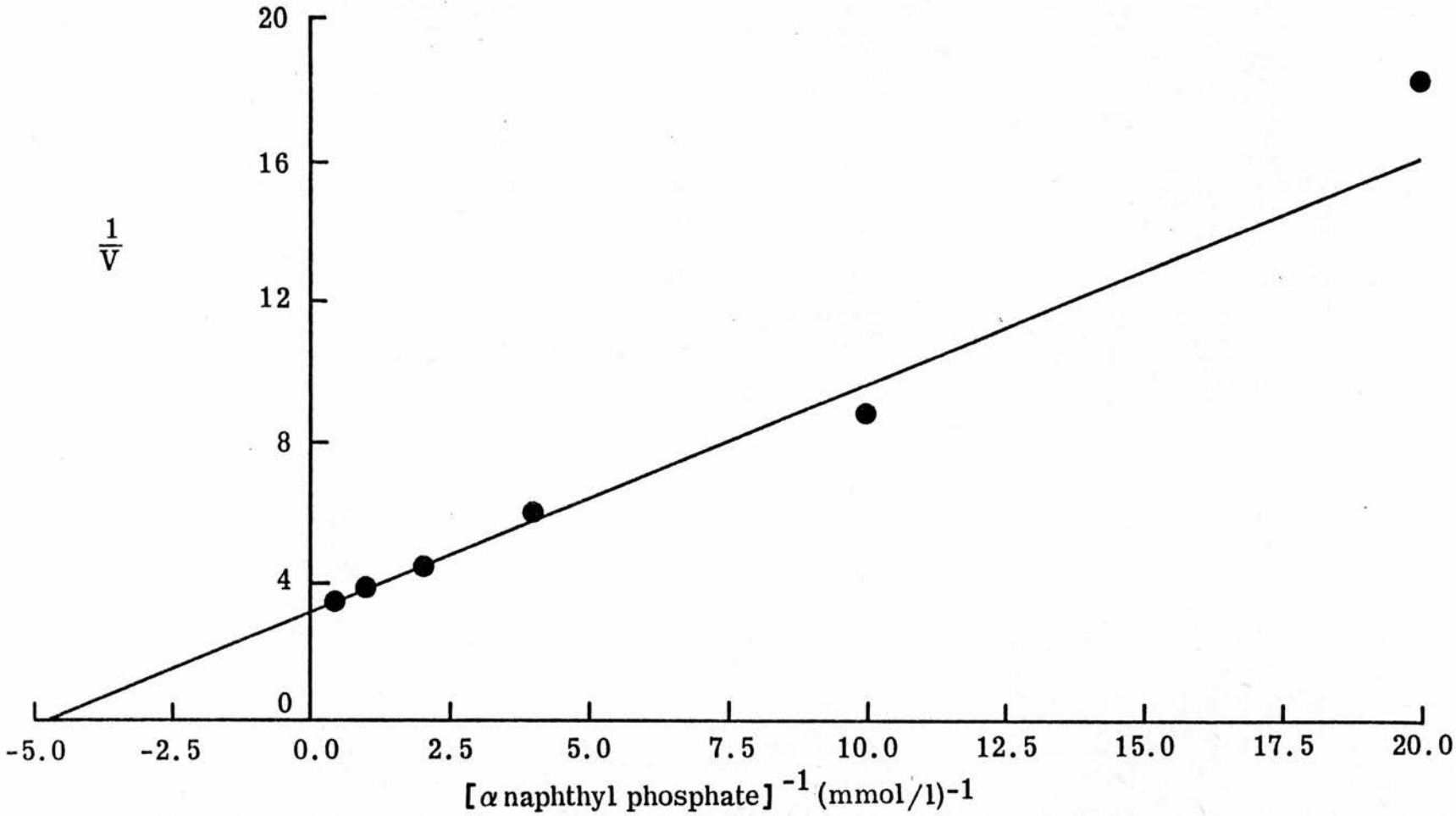


Figure 3.6. (continued).

(b) Bone alkaline phosphatase

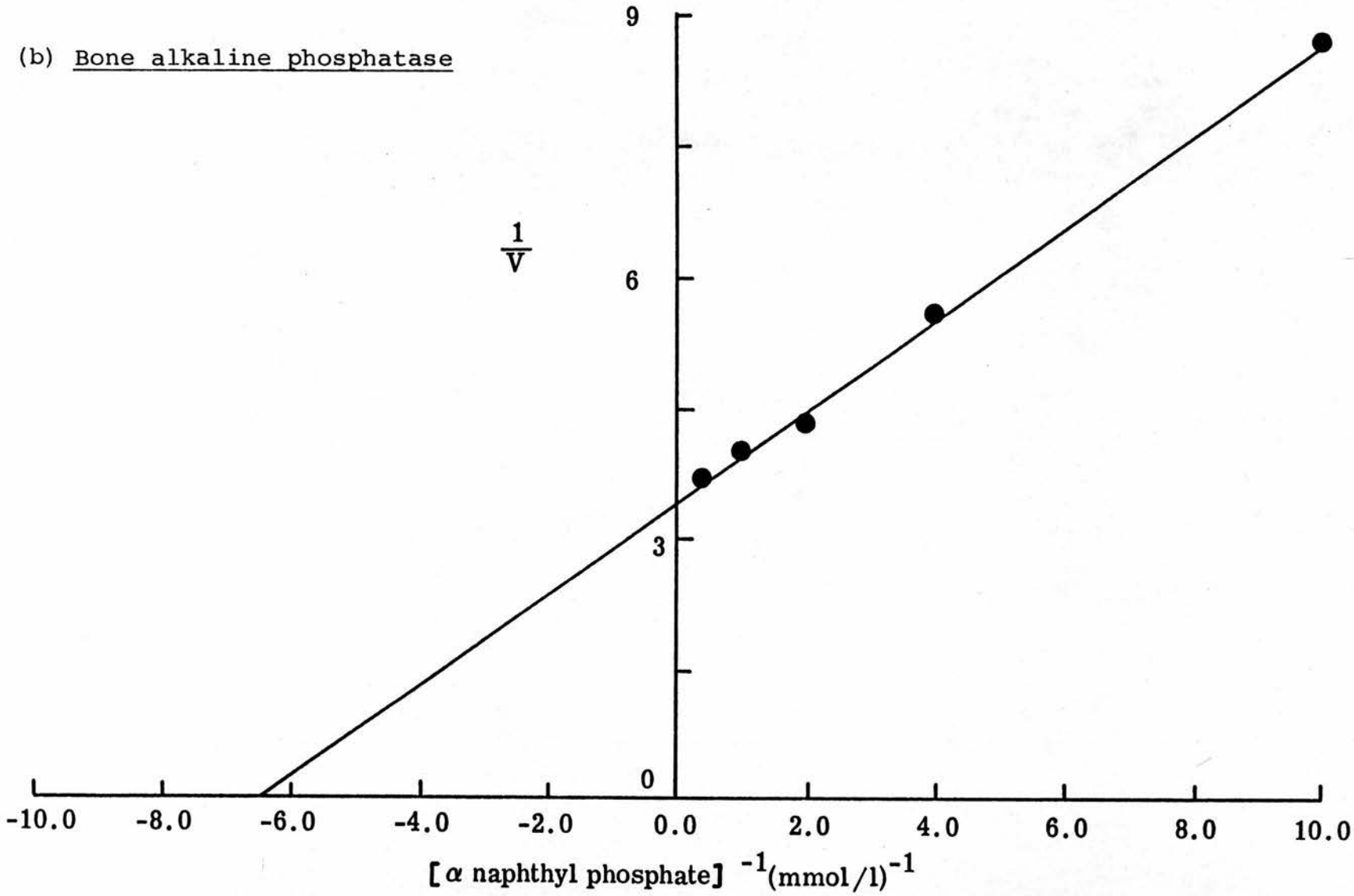


Figure 3.6. (continued).

(c) High M.W. serum alkaline phosphatase

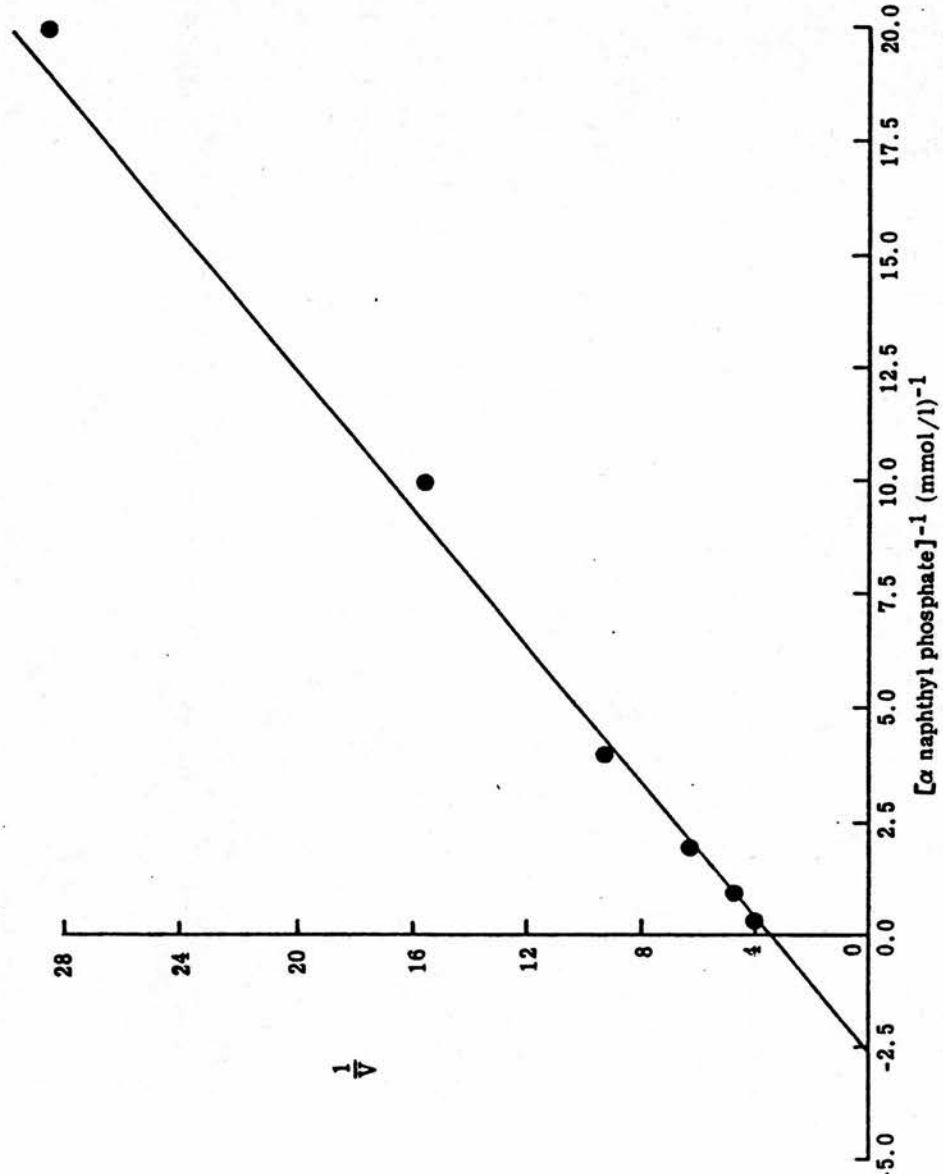


Figure 3.6. (continued)

(d) High M.W. biliary alkaline phosphatase

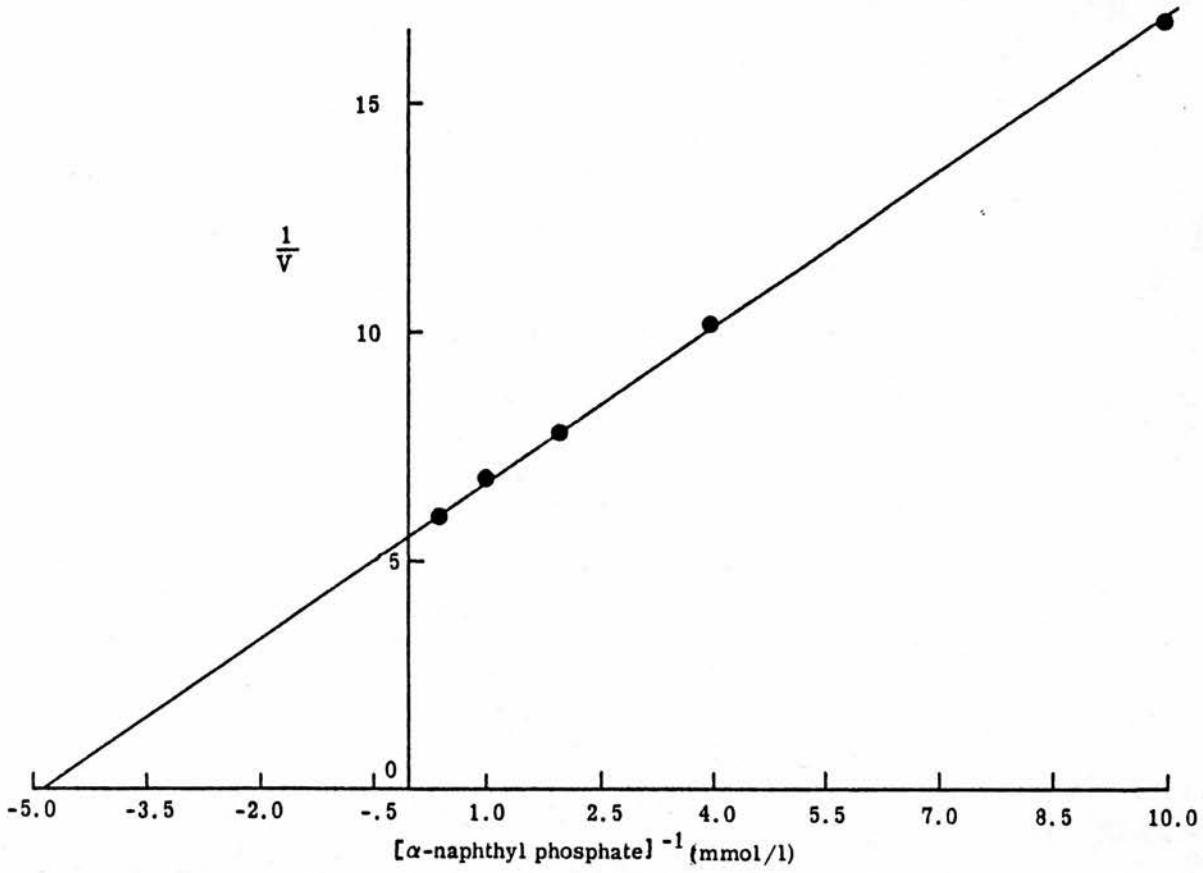
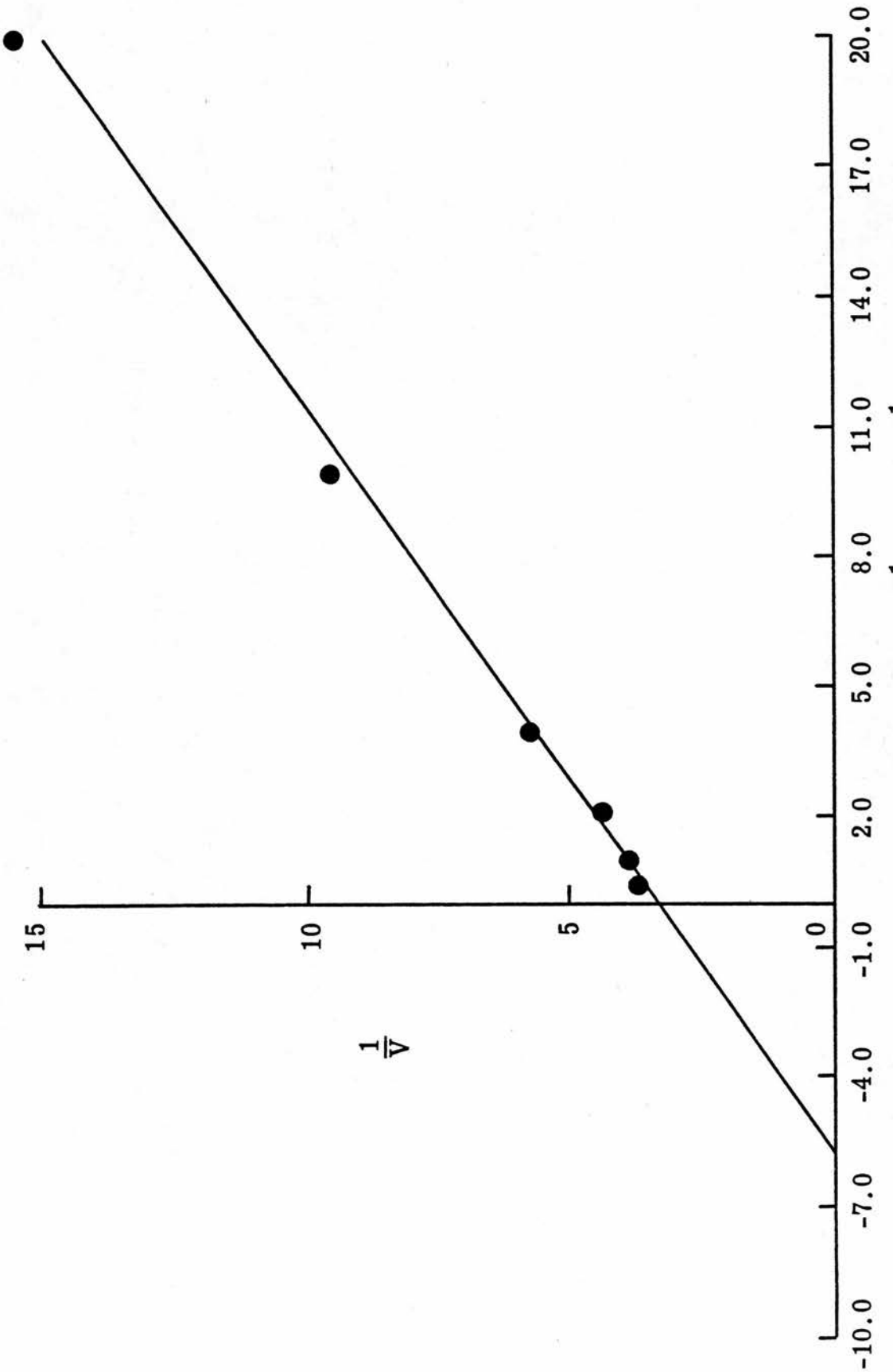


Figure 3.6. (continued).

(e) Low M.W. biliary alkaline phosphatase



were similar, though not identical, for all isoenzymes except high mol wt serum ALP. Again the K_m of the latter was approximately twice that of the other isoenzymes.

TABLE 3.3.

K_ms with respect to p-nitrophenyl phosphate as substrate.

ALP isoenzyme	Km (p-nitrophenyl phosphate) (mmol/l)	S.E. Km estimate (single data set) (mmol/l)
Liver ALP	0.84	0.08
Bone ALP	0.62	0.11
High mol wt serum ALP	1.46	0.03
High mol wt biliary ALP	0.60	0.06
Low mol wt biliary ALP	0.54	0.05

High mol wt serum ALP therefore has a lower affinity for both α-naphthyl phosphate and p-nitrophenyl phosphate than any of the other ALP isoenzymes, including high mol wt biliary ALP. This explains the discrepancy between estimates of high mol wt ALP in serum using the two substrates (section 2.8.). Routine assays used the optimised concentration of 14 mmol/l p-nitrophenyl phosphate which is on the plateau of the activity versus substrate concentration curve for all isoenzymes, including high mol wt serum ALP. The difference in affinities was therefore masked. On the

other hand, α-naphthyl phosphate was used at the sub-optimal concentration of 0.75 mmol/l which led to the difference in affinities becoming apparent. The difference in K_ms between high mol wt ALP and the other isoenzymes in serum therefore had an effect when α-naphthyl phosphate was employed as substrate but not when p-nitrophenyl phosphate was employed.

The difference in K_ms between liver and high mol wt ALP with respect to p-nitrophenyl phosphate as substrate is in conflict with the findings of Akedo et al (1967) but the purity of their preparations was considerably lower.

3.2.5. Inhibition by L-homoarginine

L-phenylalanine is the best known inhibitor of ALP (Ghosh and Fishman, 1966). It inhibits the intestinal and placental isoenzymes to a much greater extent than those from liver and bone. In the present study it was found to inhibit the liver isoenzyme by 10% and high mol wt ALP by only 4% at a concentration of 4 mmol/l. More recently inhibition of liver and bone ALP by L-homoarginine has been described (Lin and Fishman, 1972). This inhibitor was therefore studied in more detail.

The activities of the ALP isoenzymes were measured in the presence of selected concentrations of p-nitrophenyl phosphate as substrate and L-homoarginine as

inhibitor.

Fig 3.7. shows the velocity^{-1} versus [p-nitrophenyl phosphate]$^{-1}$ Lineweaver-Burk plots for each of these isoenzymes at the selected concentrations of L-homoarginine. L -homoarginine appeared to affect both K_m and V_{max}, giving parallel lines for all five ALP isoenzymes. The slopes ($= K_m/V_{max}$) for each isoenzyme individually (Table 3.4.) show no significant differences. This suggests that the inhibition was of the uncompetitive type for all the isoenzymes.

In uncompetitive inhibition, the enzyme-inhibitor complex is not formed. Instead the inhibitor can only combine with the enzyme-substrate intermediate, resulting in a complex which does not break down. This may be summarised by the following equilibria:

$$E + S \rightleftharpoons ES \qquad (1)$$

$$ES + I \rightleftharpoons EIS \qquad (2)$$

$$ES \longrightarrow E + P \qquad (3)$$

If e is the enzyme (E) concentration, s the substrate (S) concentration, i the inhibitor (I) concentration, p the enzyme-substrate intermediate (ES) concentration, q the enzyme-substrate-inhibitor complex (EIS) concentration, K_m the dissociation constant of ES, K_i the dissociation constant of EIS, v the velocity of the reaction in equation

Figure 3.7. Lineweaver-Burk plots of 1/v versus [p-nitrophenyl phosphate]$^{-1}$ at selected concentrations of L-homoarginine for each ALP preparation. V is measured as μmoles p-nitrophenol produced per min per litre. —●— 0; —▲— 2; —○— 4; —△— 6 mmol/l L-homoarginine.

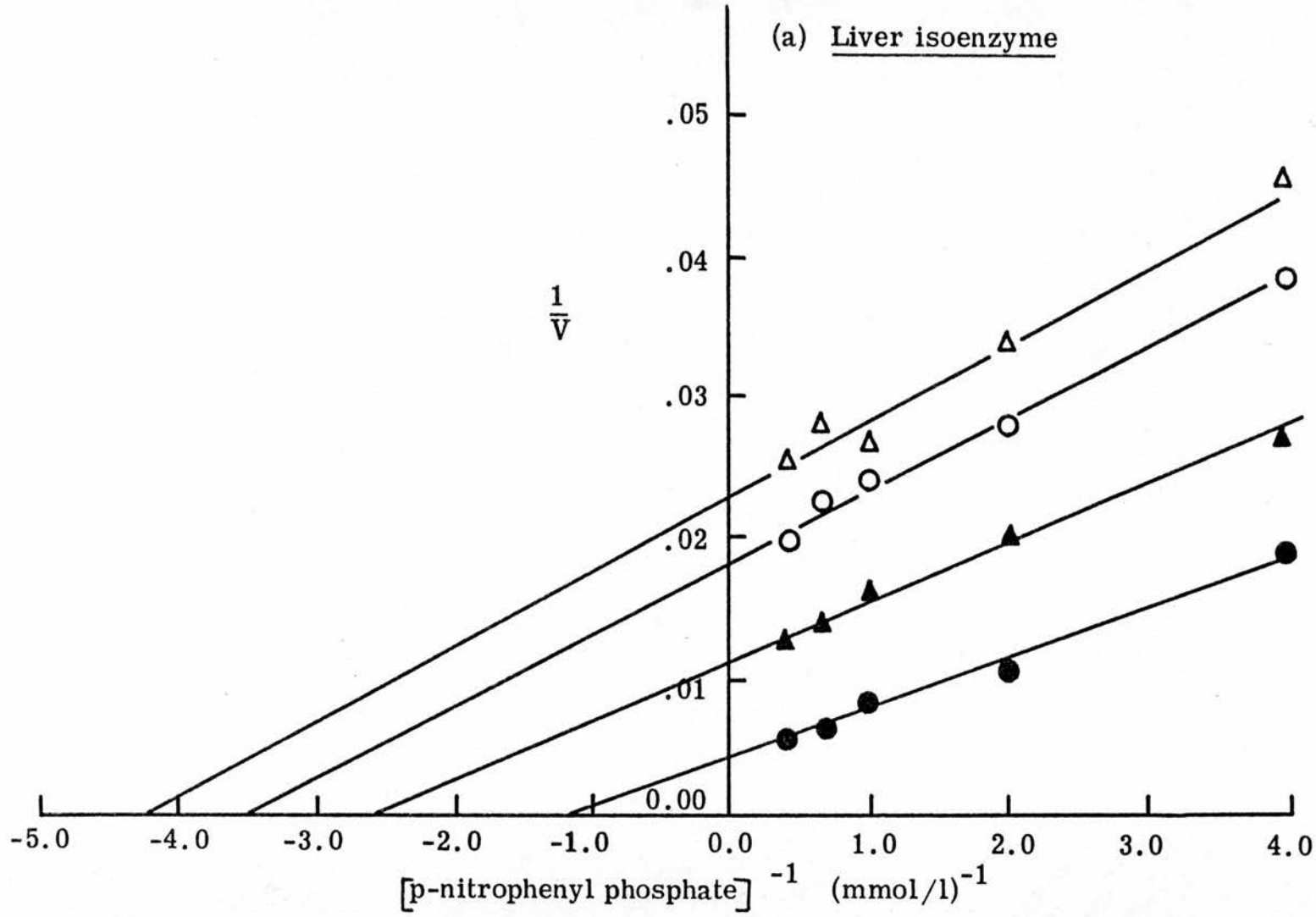
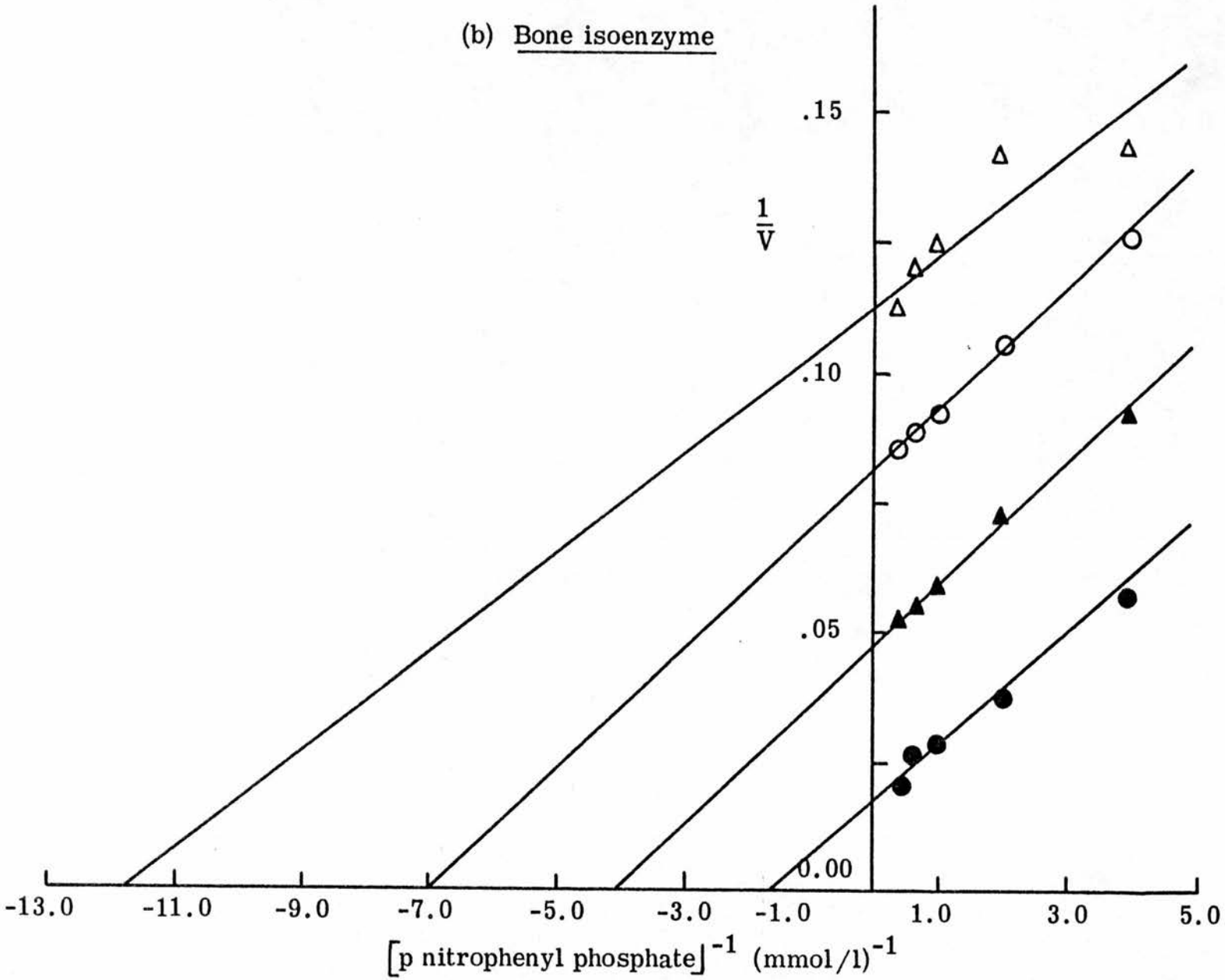


Figure 3.7. (continued).



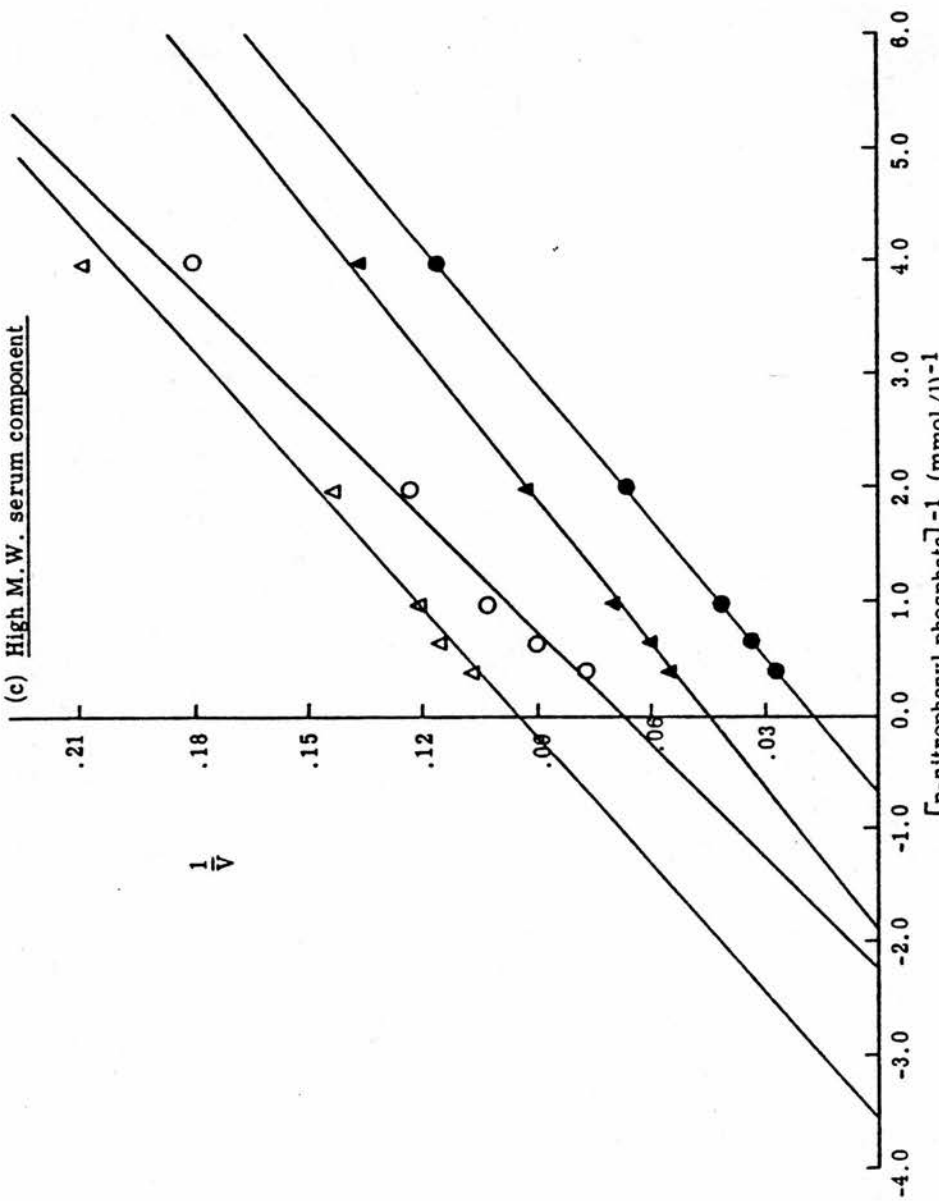
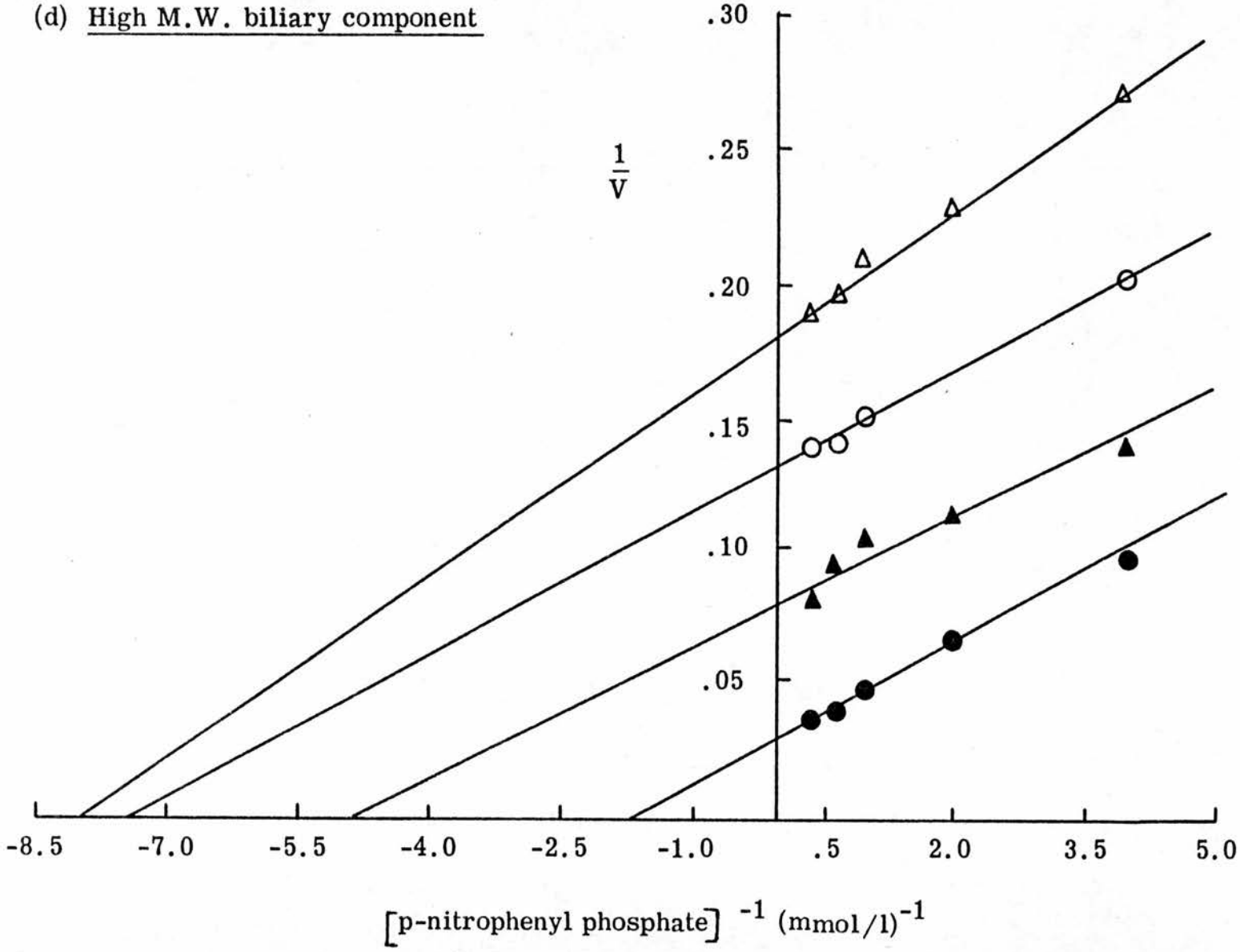


Figure 3.7. (continued).

Figure 3.7. (continued).

(d) High M.W. biliary component



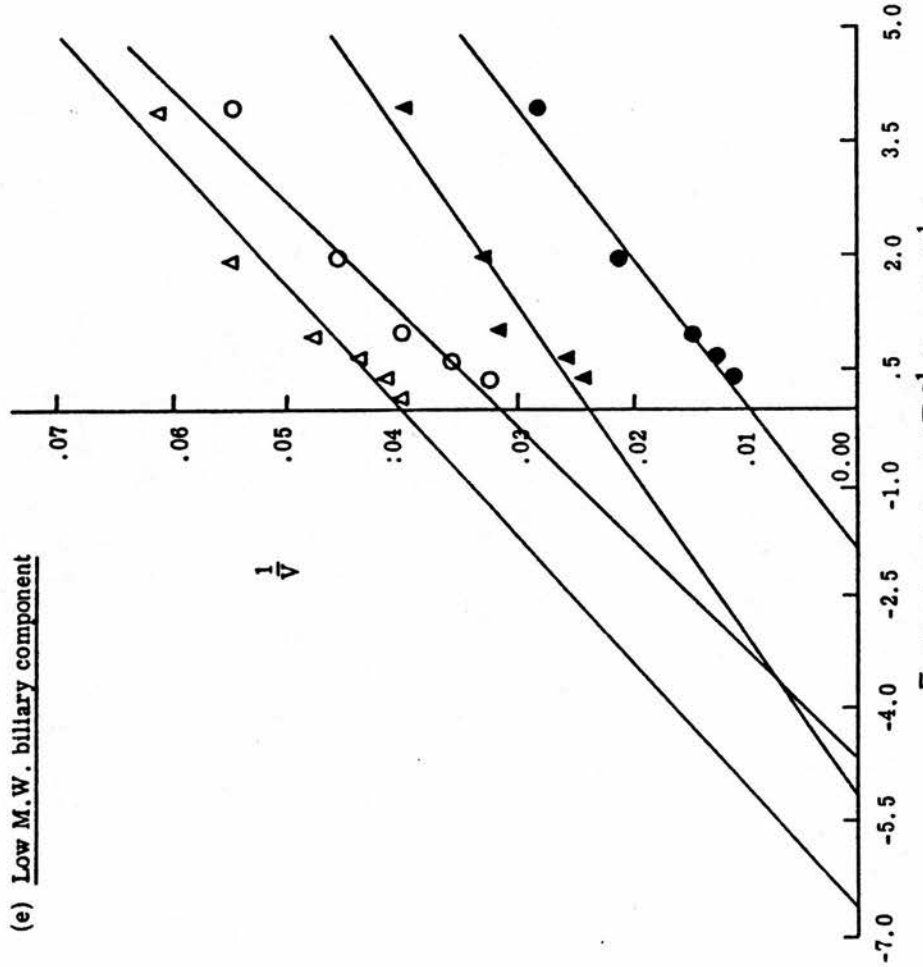


Figure 3.7. (continued).

TABLE 3.4.

Slopes of Lineweaver-Burk plots at various concentrations of L-homoarginine

ALP isoenzyme	[L-homoarginine] (mmol/l)	Slope (K_m/V_{max}) ($\times 10^{-3}$)	S.E.(slope) * ($\times 10^{-3}$)
Liver ALP	0	3.5	0.3
	2	4.3	0.3
	4	5.1	0.6
	6	5.3	1.0
Bone ALP	0	10.7	1.9
	2	11.7	0.6
	4	11.6	0.3
	6	9.6	3.2
High mol wt serum ALP	0	25	1
	2	24	1
	4	30	5
	6	26	3
High mol wt biliary ALP	0	18	2
	2	17	4
	4	18	1
	6	23	2
Low mol wt biliary ALP	0	5.1	0.5
	2	4.6	1.4
	4	6.7	1.4
	6	6.0	1.1

* Calculated from the formula:
$(S.E.slope)^2 = (S.E.K_m)^2 + (S.E.V_{max})^2$

(3) and k the velocity constant for equation (3), the following equations can be set up by the Law of Mass Action:

$$(e - p - q)s = K_m p \quad \text{————} \quad (4)$$

$$pi = K_i q \quad \text{————} \quad (5)$$

$$v = kp \quad \text{————} \quad (6)$$

Solving to eliminate the unknowns, p and q, gives:

$$v = \frac{ke}{K_{m/s} + (1 + \frac{i}{K_i})}$$

Now $ke = V_{max}$ since under these circumstances all the enzyme would be involved in the ES complex.

Therefore, $$v = \frac{V_{max}}{K_{m/s} + (1 + \frac{i}{K_i})}$$

Rearranging, $$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{s} + \frac{1 + {}^{i}/K_i}{V_{max}}$$

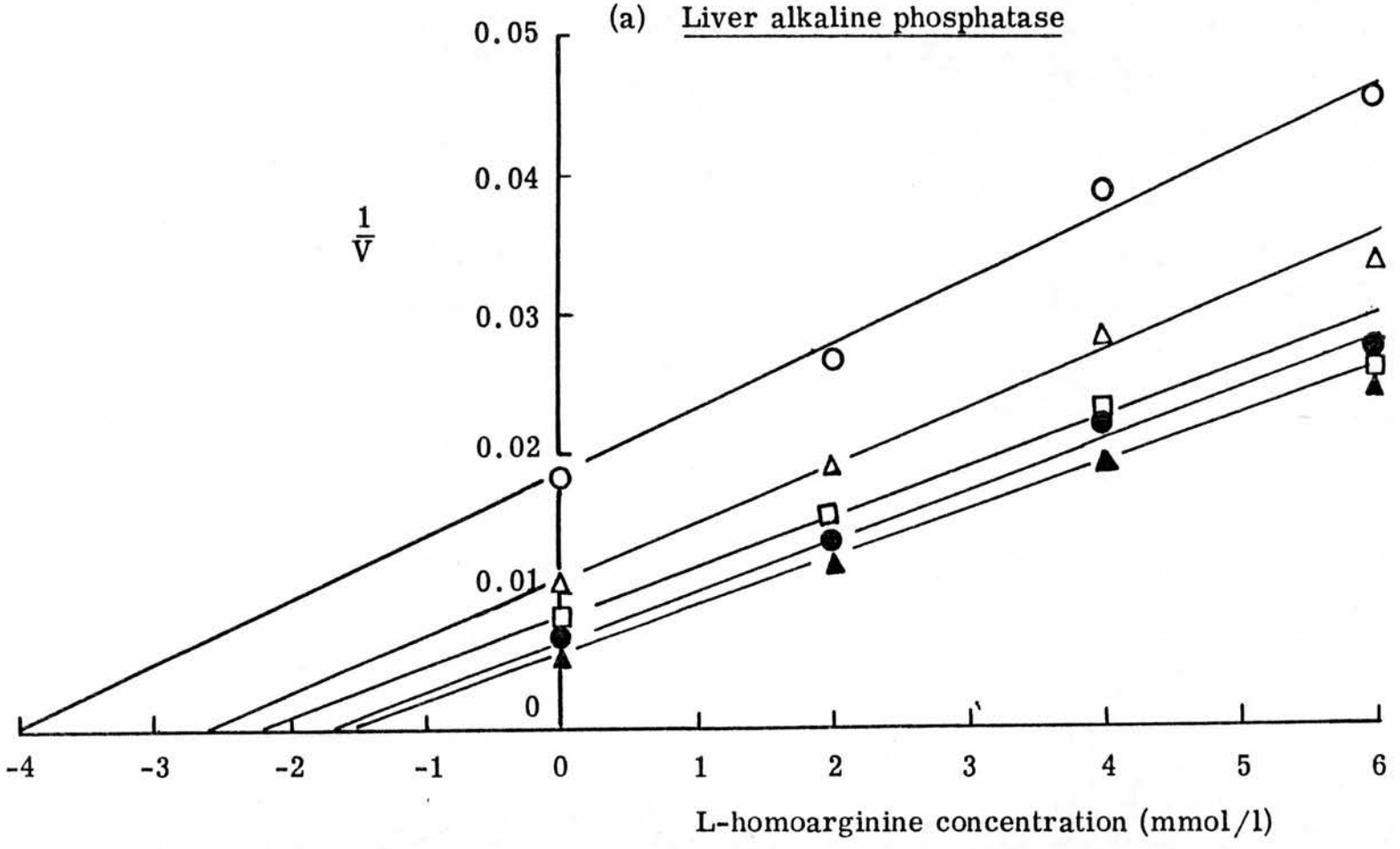
Therefore the usual Lineweaver-Burk plots of $^{1}/v$ versus $^{1}/s$ will result in a series of parallel lines at all inhibitor concentrations with a slope of $^{K_m}/V_{max}$.

Rearranging again, $$\frac{1}{v} = \frac{1}{V_{max}} (1 + {}^{K_m}/_s) + \frac{1}{K_i V_{max}} \cdot i$$

Therefore a plot of $^{1}/v$ against i will also result in a series of parallel lines at all substrate concentrations with a slope of $^{1}/K_i V_{max}$, from which K_i can be calculated.

Therefore, in order to confirm the uncompetitive nature of the inhibitors and calculate K_i, velocity^{-1} was

Figure 3.8. 1/v versus [L-homoarginine] plots at selected concentrations of p-nitrophenyl phosphate for each ALP preparation. V is measured as μ moles p-nitrophenol produced per min per litre. —▲— 0.25; —●— 0.5; —□— 1.0; —△— 1.5; —○— 2.5 mmol/l p-nitrophenyl phosphate.



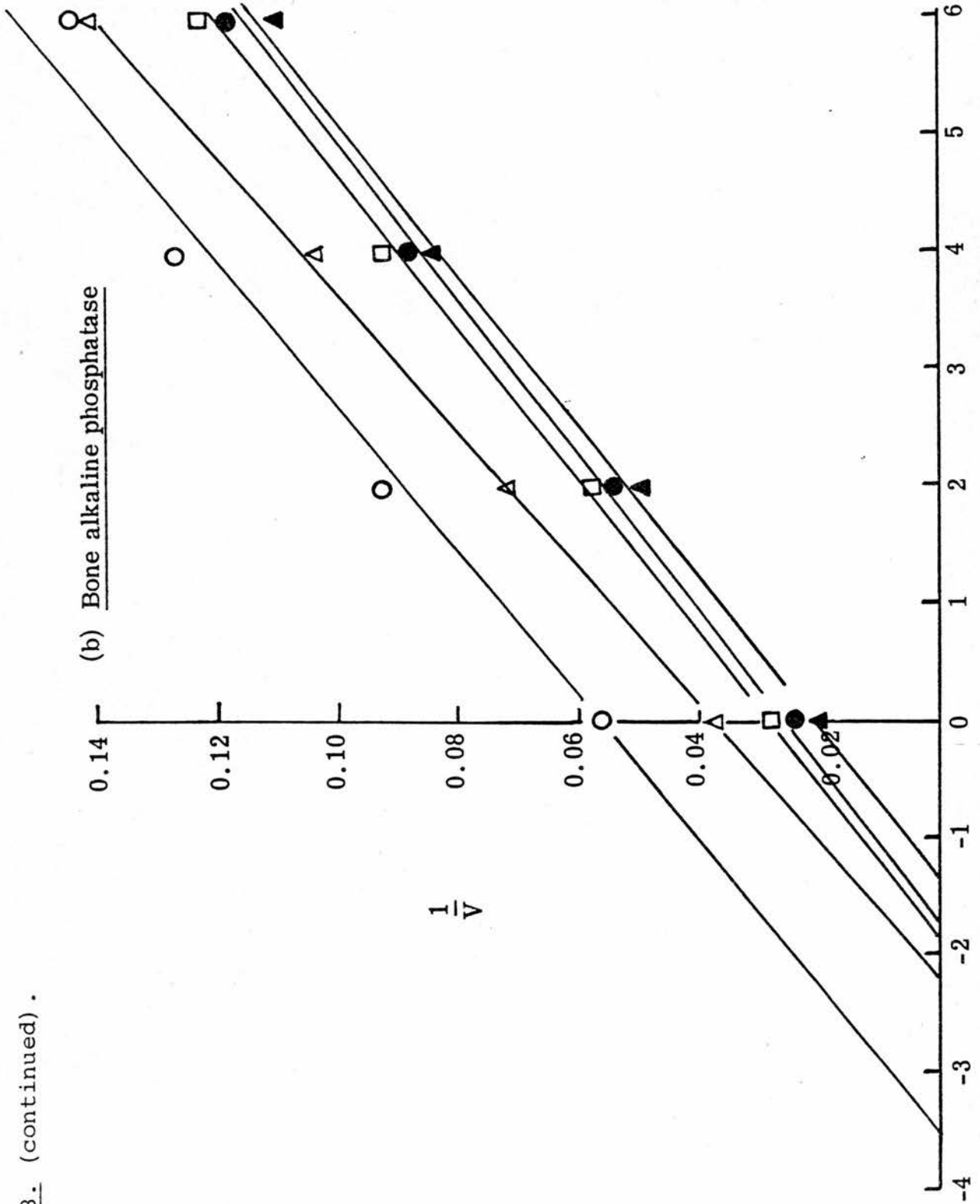


Figure 3.8. (continued).

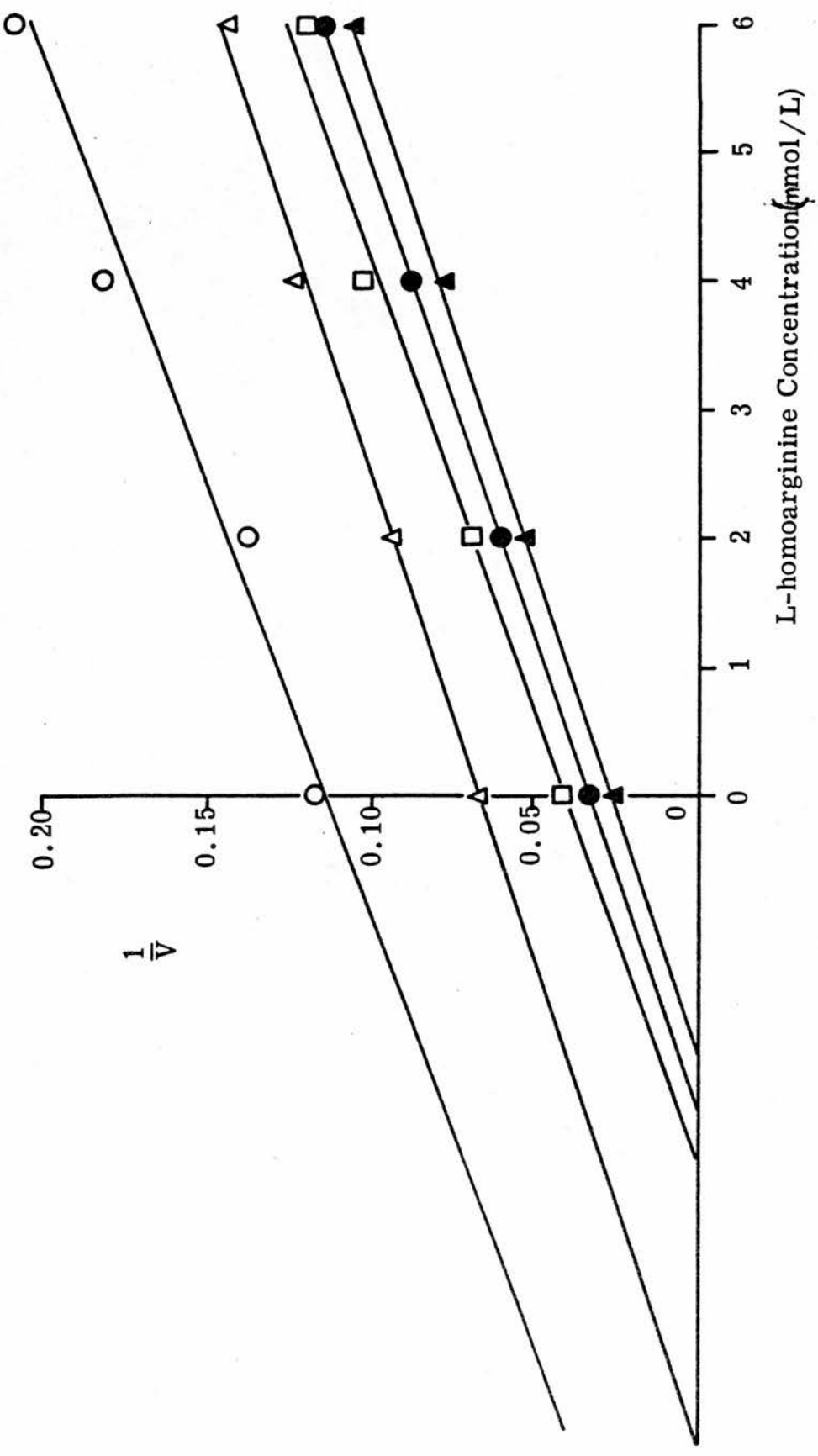


Figure 3.8. (continued).

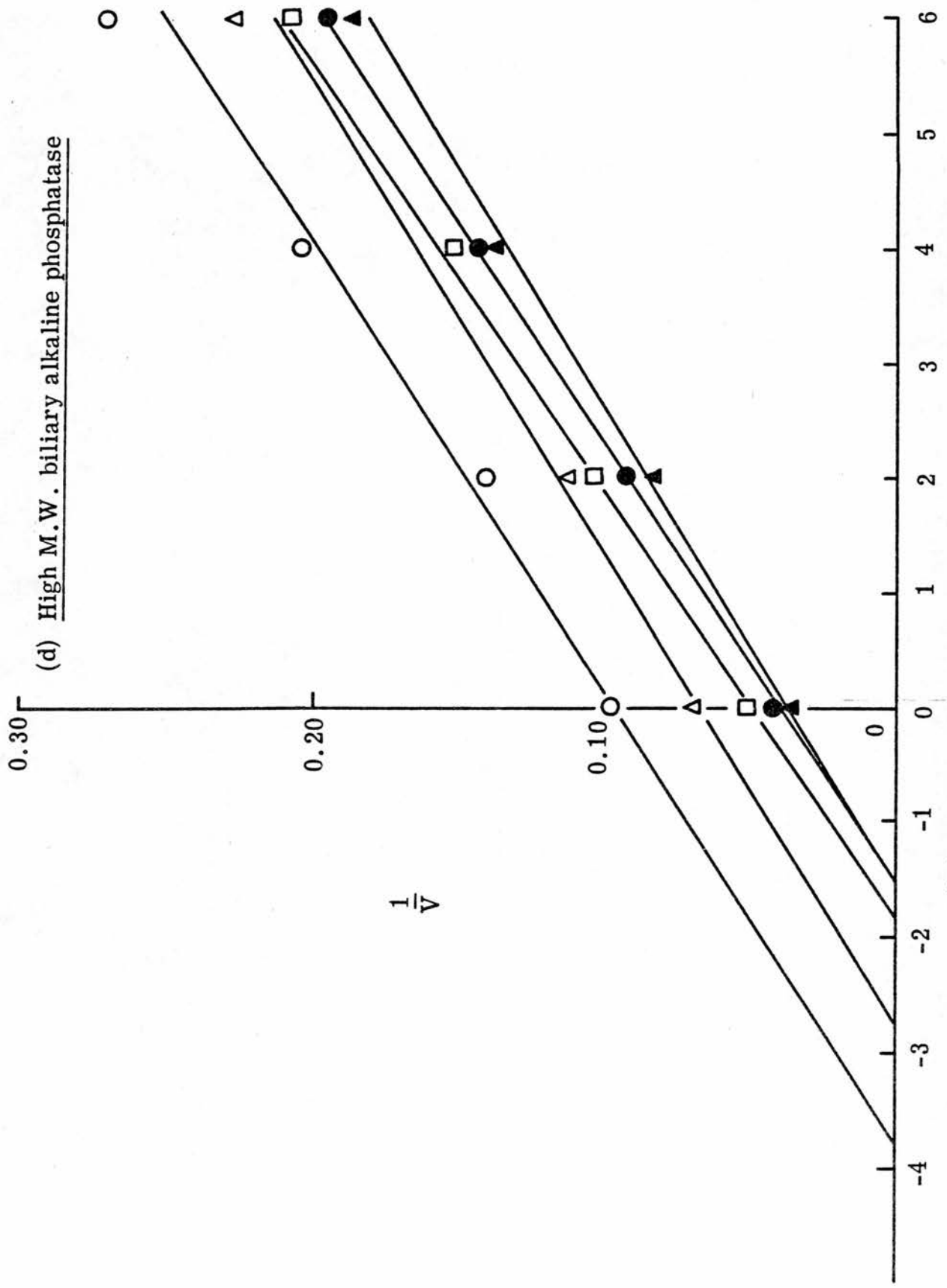


Figure 3.8. (continued).

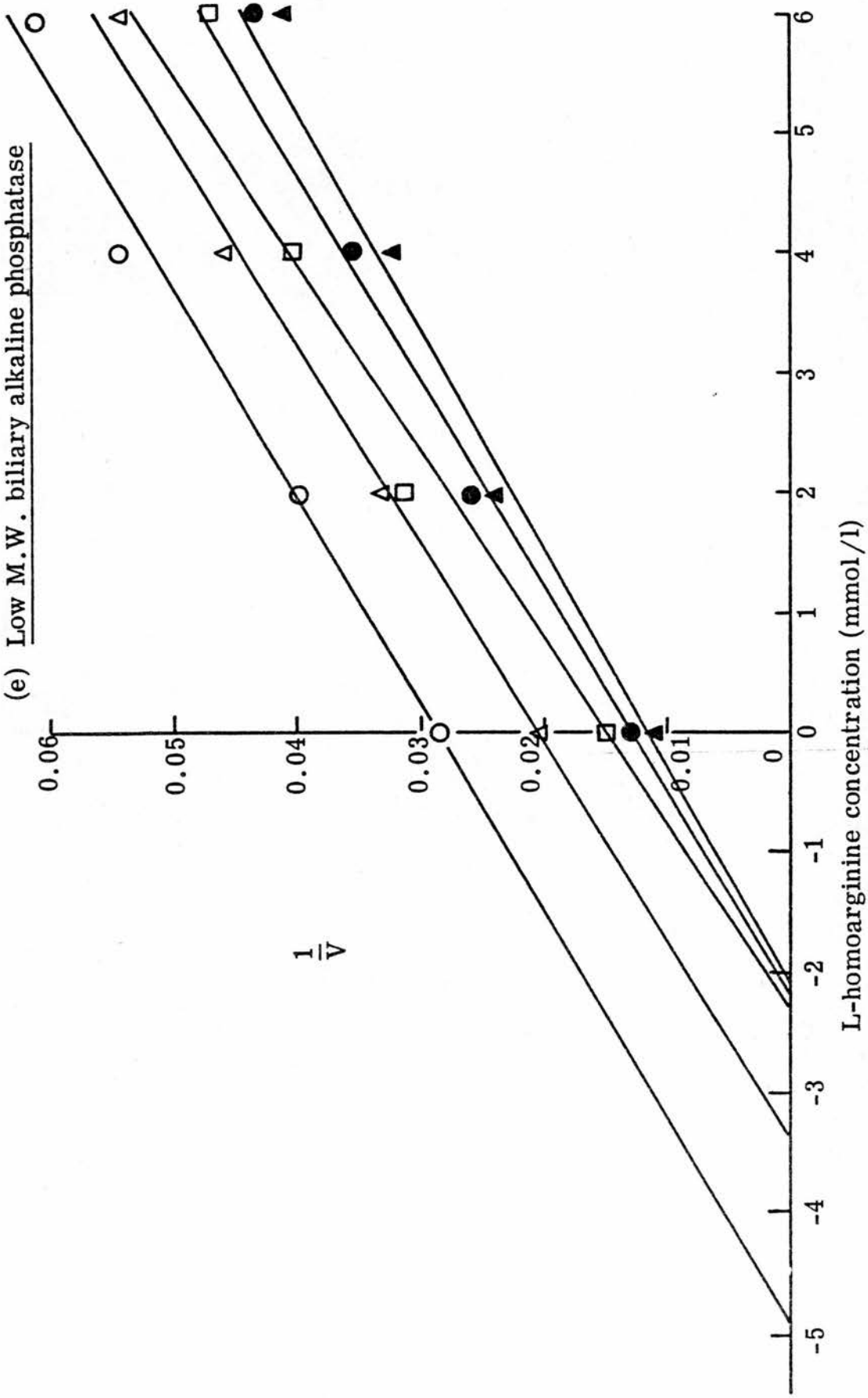


Figure 3.8. (continued).

TABLE 3.5.

Slopes of velocity^{-1} versus [L-homoarginine] plots at various concentrations of p-nitrophenyl phosphate

ALP isoenzyme	[p-nitrophenyl phosphate] (mmol/l)	Slope ($\times 10^{-3}$)	S.E. (slope) ($\times 10^{-3}$)
Liver ALP	0.25	4.6	1.3
	0.5	4.1	0.3
	1.0	3.6	0.3
	1.5	3.7	0.1
	2.5	3.4	0.1
Bone ALP	0.25	16.1	1.2
	0.5	17.0	0.1
	1.0	15.4	0.4
	1.5	15.0	0.4
	2.5	15.7	0.3
High mol wt serum ALP	0.25	15.0	2.0
	0.5	13.3	0.5
	1.0	14.1	0.8
	1.5	13.5	0.2
	2.5	13.2	0.4
High mol wt biliary ALP	0.25	25.4	2.4
	0.5	24.1	2.0
	1.0	26.6	0.7
	1.5	26.0	0.4
	2.5	23.8	1.1
Low mol wt biliary ALP	0.25	5.8	0.4
	0.5	6.0	0.2
	1.0	6.4	0.8
	1.5	5.8	0.4
	2.5	5.5	0.4

plotted against L-homoarginine concentration for each concentration of p-nitrophenyl phosphate (Fig 3.8.). Table 3.5. shows the slopes with their standard errors, confirming that the lines were parallel.

TABLE 3.6.

Estimates of K_i derived from velocity^{-1} versus [L-homoarginine] plots

ALP Isoenzyme	mean K_i (n=5) (mmol/l)	S.D. K_i (mmol/l)
Liver ALP	1.1	0.13
Bone ALP	1.1	0.05
High mol wt serum ALP	1.2	0.06
High mol wt biliary ALP	1.2	0.06
Low mol wt biliary ALP	1.6	0.09

Table 3.6. shows the mean and S.D. of the estimates of K_i derived from these slopes for each isoenzyme. One way analysis of variance showed that there was at least one isoenzyme which differed significantly from the others ($p < 0.001$). The K_i of the low mol wt biliary isoenzyme was found to be significantly different from the other K_is ($p < 0.001$). The K_is of the high mol wt ALPs from serum and bile were slightly but significantly higher than the K_is of the liver and bone isoenzymes ($p < 0.05$). However, this

slight difference could be explained by the higher degree of purity attained for the high mol wt ALPs and is in any case too small to indicate a basic difference between the isoenzymes.

In conclusion, with respect to inhibition by L-homoarginine the high mol wt ALPs from serum and bile resemble each other and also the liver and bone isoenzymes.

3.2.6. Activation by magnesium ions

Although it is generally recognised that ALP is activated by magnesium ions (Mg^{2+}) no detailed mechanism has been worked out. It is thought that the binding of substrate and of Mg^{2+} to the enzyme are two independent events and that Mg^{2+} must therefore play a part in substrate decomposition (Ahlers, 1974). This may be summarised by the following equilibria, using the same notation as in section 3.2.5.

$$E + Mg^{2+} \underset{}{\overset{K_A}{\rightleftharpoons}} EMg^{2+} \qquad (1)$$

$$E + S \overset{K_m}{\rightleftharpoons} ES \qquad (2)$$

$$EMg^{2+} + S \overset{K_m}{\rightleftharpoons} EMg^{2+}S \qquad (3)$$

$$ES + Mg^{2+} \overset{K_A}{\rightleftharpoons} EMg^{2+}S \qquad (4)$$

$$EMg^{2+}S \xrightarrow{v} EMg^{2+} + \text{products} \qquad (5)$$

If a is the concentration of Mg^{2+}, p the concentration of

ES, q the concentration of $EMg^{2+}S$, r the concentration of EMg^{2+} and K_A the dissociation constant of EMg^{2+}, then the following equations may be set up by the Law of Mass Action:

$$(e - p - q - r)a = K_A r \quad \text{——————} \quad (6)$$

$$(e - p - q - r)s = K_m p \quad \text{——————} \quad (7)$$

$$rs = K_m q \quad \text{——————} \quad (8)$$

$$pa = K_A q \quad \text{——————} \quad (9)$$

$$v = kq \quad \text{——————} \quad (10)$$

Solving these for p, q and r, we obtain:

$$v = \frac{ke}{(1 + \frac{K_m}{s})(1 + \frac{K_A}{a})} \quad \text{——————} \quad (11)$$

which is symmetrical for substrate and activator.

Now, let $V_a = \frac{ke}{1 + \frac{K_m}{s}}$

Then from (11), $v = \frac{V_a}{(1 + \frac{K_A}{a})}$

Rearranging, $\frac{1}{v} = \frac{1}{V_a} + \frac{K_A}{V_a} \cdot \frac{1}{a}$

and a 1/v versus a 1/a plot will give a negative intercept on the 1/a axis of $1/K_A$. This intercept is independent of variations in substrate concentration. Since equation (11) is symmetrical for substrate and activator, a 1/v versus 1/S plot will also give a negative intercept on the 1/S axis of $1/K_m$. This intercept is also independent of

variations in magnesium concentration.

The activities of liver and high mol wt serum ALP were measured in 1.5 mol/l DEA-HCl buffer pH 10.2 at various concentrations of p-nitrophenyl phosphate and at various concentrations of added magnesium chloride.

Fig 3.9. shows the double reciprocal plots of reaction velocity versus p-nitrophenyl phosphate concentration at selected Mg^{2+} concentrations. The K_m did not seem to be significantly affected by Mg^{2+} concentration but there was an increase in V_{max} as Mg^{2+} concentration increased (Table 3.7.). This is in agreement with the mechanism for activation presented above.

It is interesting to note that in this experiment the K_m's of liver and high mol wt ALP appeared to be similar at most Mg^{2+} concentrations, contrary to the findings in section 3.2.4. The explanation may be that the differences only manifested themselves at much higher Mg^{2+} concentrations i.e. 500 μmol/l rather than the maximum of 50 μmol/l in this experiment.

The endogenous Mg^{2+} content of the buffered enzyme preparations was measured by atomic absorption spectrophotometry and found to be 13 μmol/l for the liver isoenzyme and 8 μmol/l for high mol wt ALP. A further 6 μmol/l was contributed by a 10 mmol/l solution of

Figure 3.9. Lineweaver-Burk plots of $1/v$ versus $[\text{p-nitrophenyl phosphate}]^{-1}$ at selected concentrations of magnesium ions. V is measured as μmoles p-nitrophenol produced per min per litre. —□— 0; —△— 5; —○— 10; —▲— 20; —●— 50 μmol/l added magnesium ions.

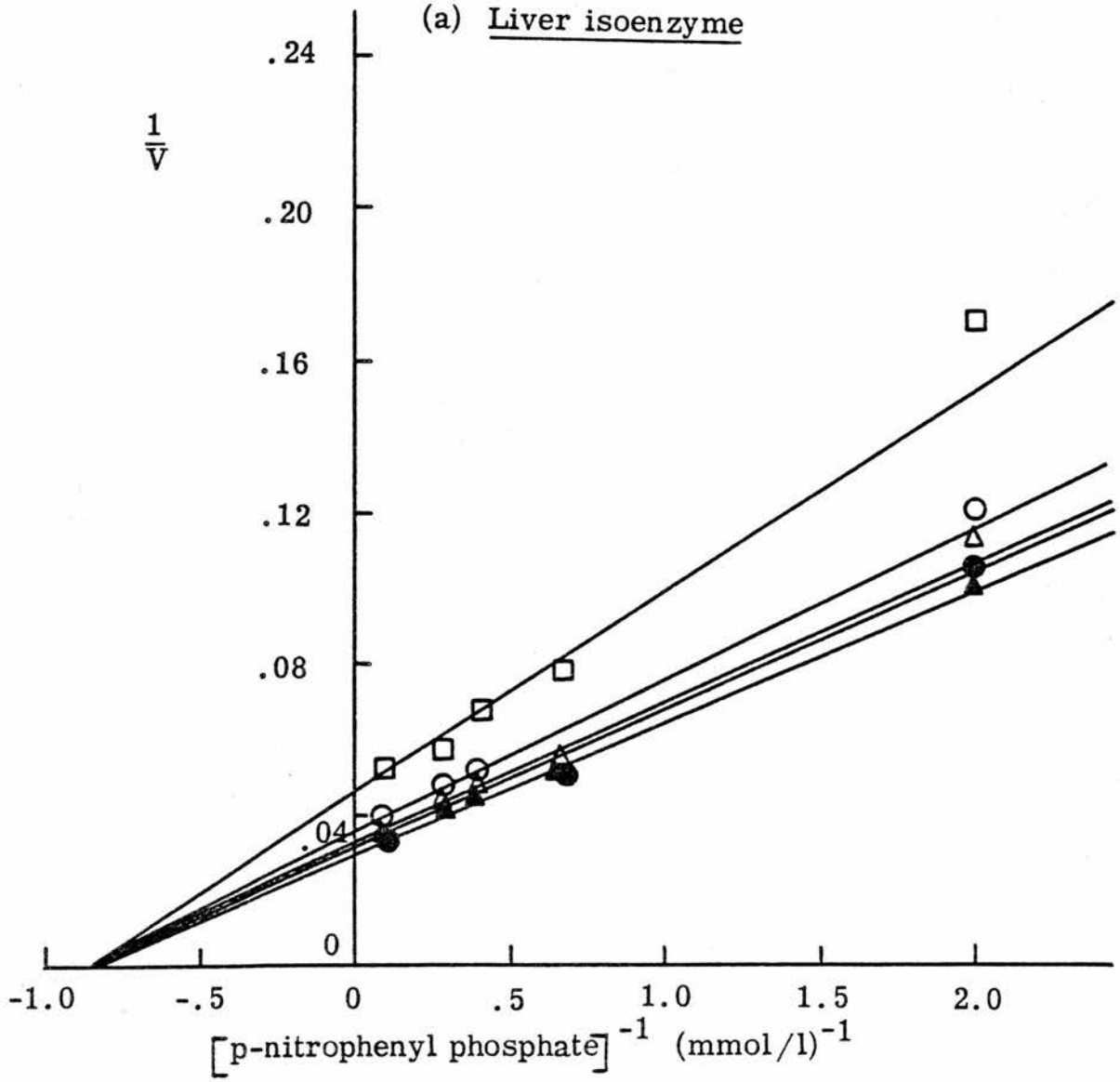


Figure 3.9. (continued).

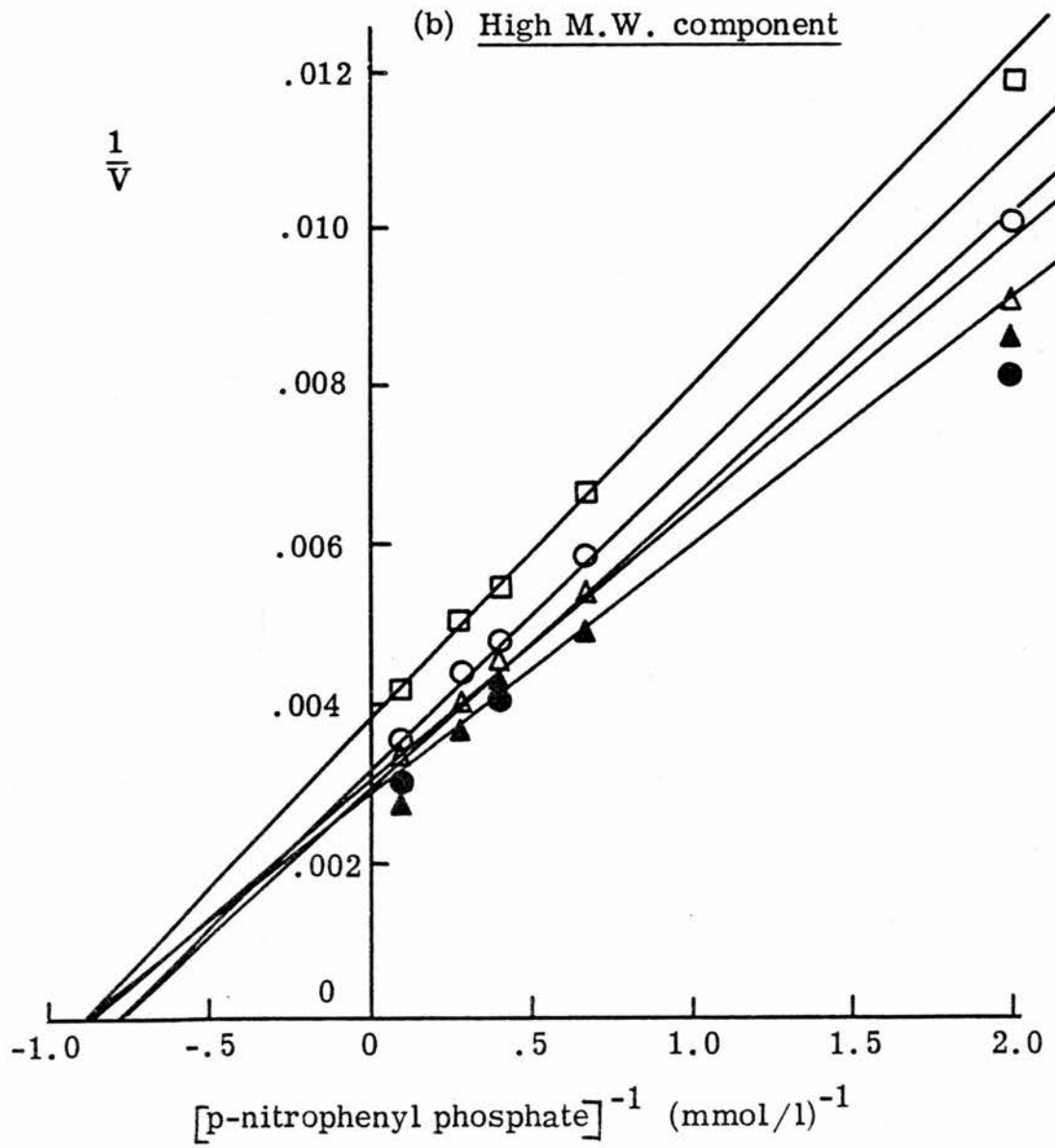


TABLE 3.7.

Effect of magnesium ions on K_m and V_{max}

Concentration of added Mg^{2+} (μmol/l)	K_m (S.E.) (mmol/l)		V_{max} (S.E.) (iu/l)	
	Liver ALP	High mol wt ALP	Liver ALP	High mol wt ALP
0	1.17 (0.17)	1.13 (0.04)	2211 (96)	268 (3)
5	1.13 (0.08)	1.28 (0.13)	2831 (58)	323 (10)
10	1.10 (0.17)	1.17 (0.10)	3023 (93)	338 (9)
20	1.19 (0.10)	1.17 (0.20)	3275 (85)	362 (19)
50	1.07 (0.08)	1.63 (0.31)	3193 (68)	421 (27)

p-nitrophenyl phosphate. Correcting the concentration of added Mg^{2+}, the velocity versus Mg^{2+} concentration plots shown in Fig 3.10. were obtained.

It is apparent from Fig 3.10 that the fit would be improved if the origin were adjusted along the $[Mg^{2+}]$ axis. This procedure could be rationalised by the hypothesis that not all the endogenous Mg^{2+} ions were available to the enzyme: some might be inaccessibly bound to contaminating protein in the partially purified preparations.

On an empirical basis it was found that minimum residual variance of the experimental points from the fitted curve was obtained by assuming an available endogenous Mg^{2+} ion concentration of 2 μmol/l in the buffered liver isoenzyme preparation and 4 μmol/l in the buffered high mol wt ALP preparation (Fig 3.11.). The double reciprocal plots of reaction velocity versus Mg^{2+} concentration using these modified data are shown in Fig 3.12.

The apparent K_A, defined as the reciprocal of the intercept on the $[Mg^{2+}]^{-1}$ axis, is shown in Table 3.8. for various concentrations of substrate. If liver and high mol wt ALP are considered separately, there appears to be little significant variation in the value of the apparent K_A with variations in substrate concentration. Again this is in agreement with the mechanism for activation presented

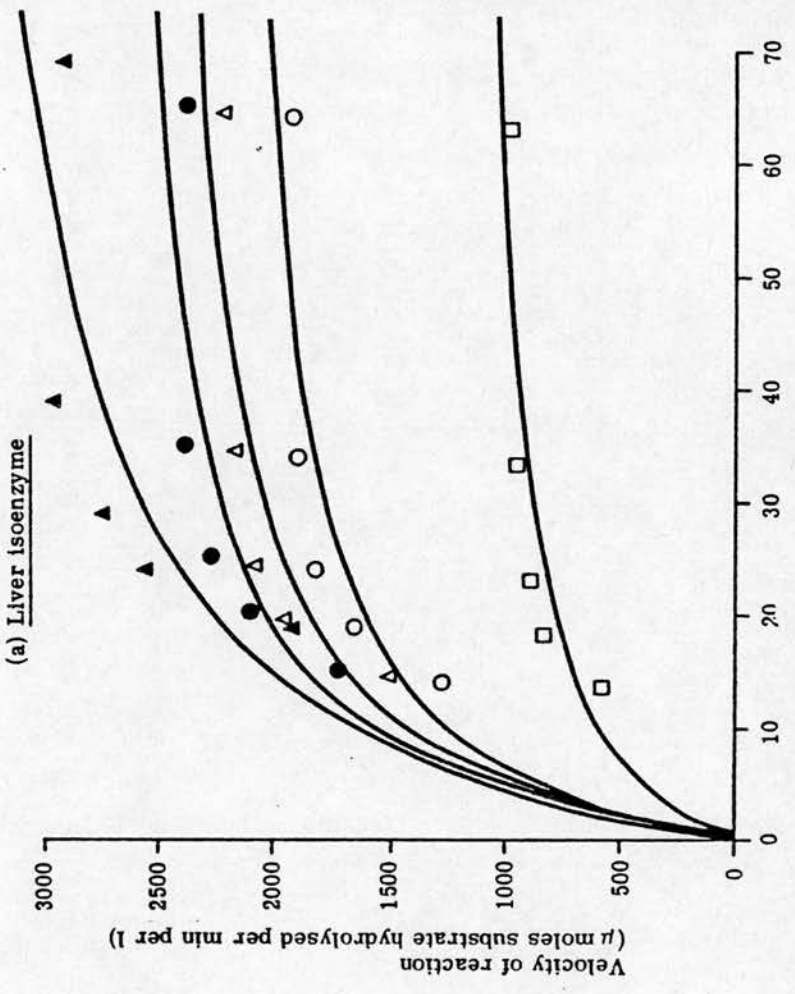


Figure 3.10. Plots of velocity versus total measured magnesium concentration at selected concentrations of p-nitrophenyl phosphate. —□— 0.5; —○— 1.5; —△— 2.5; —●— 3.5; —▲— 10.0 mmol/l p-nitrophenyl phosphate.

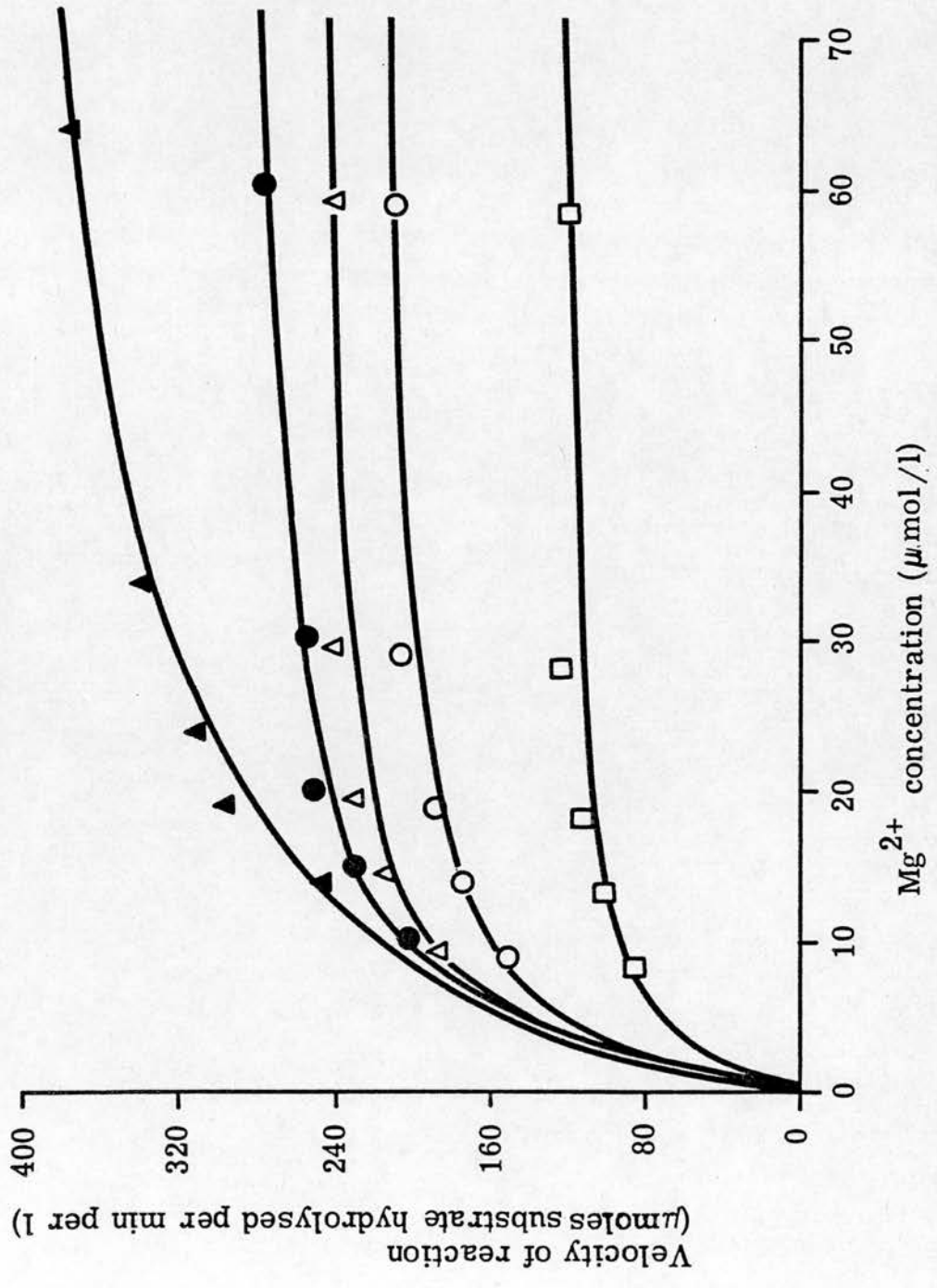


Figure 3.10. (continued).

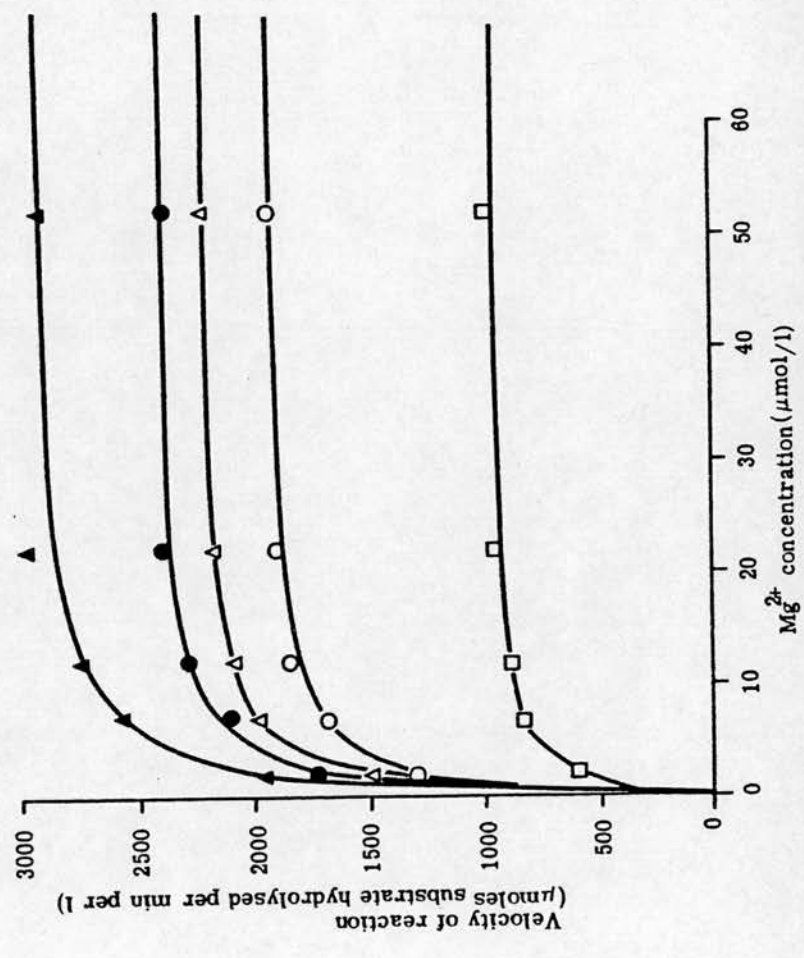


Figure 3.11. Plots of velocity versus "best fit" magnesium concentration (see text) at selected concentrations of p-nitrophenyl phosphate. —□— 0.5; —○— 1.5; —△— 2.5; —●— 3.5; —▲— 10.0 mmol/l p-nitrophenyl phosphate.

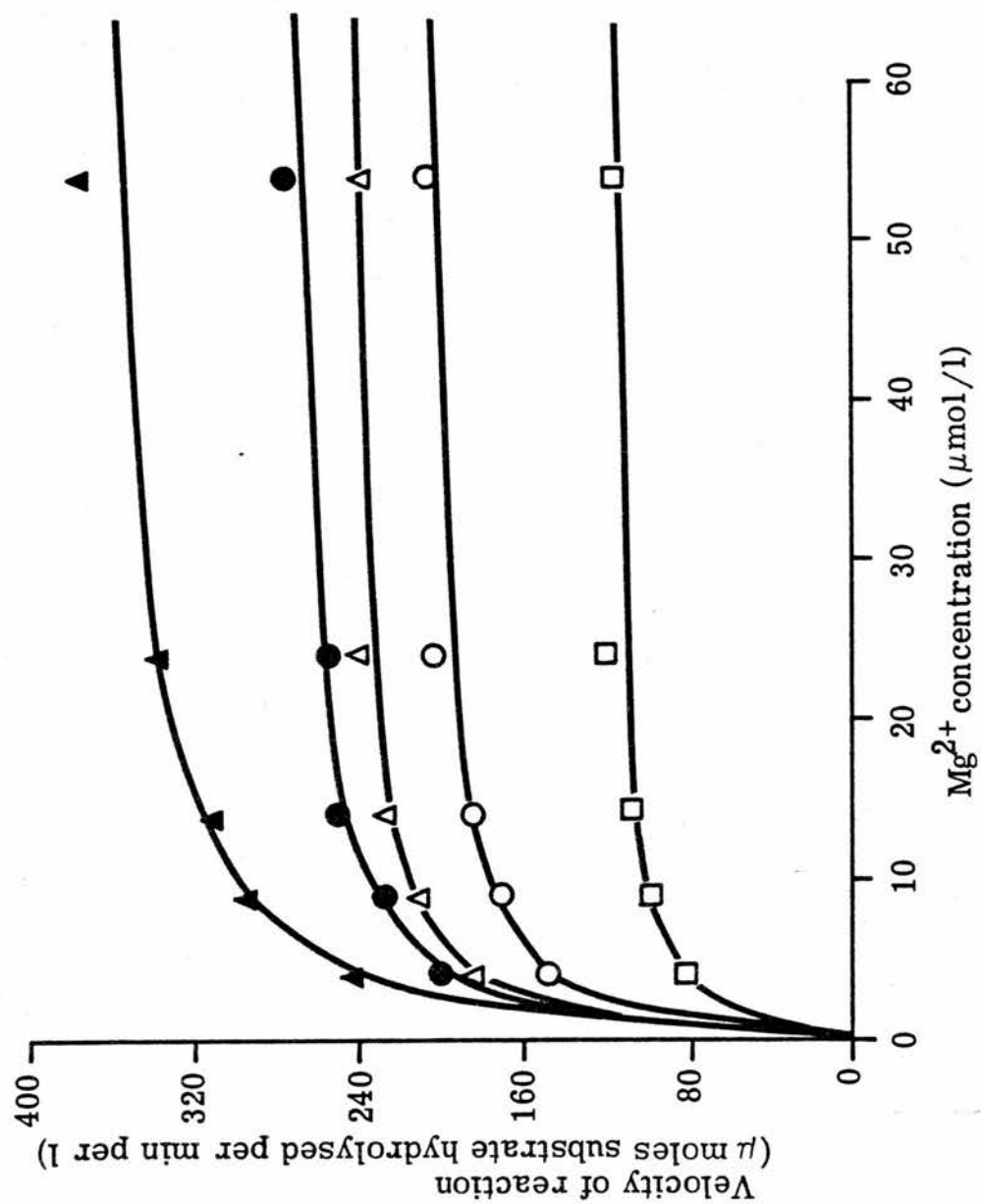
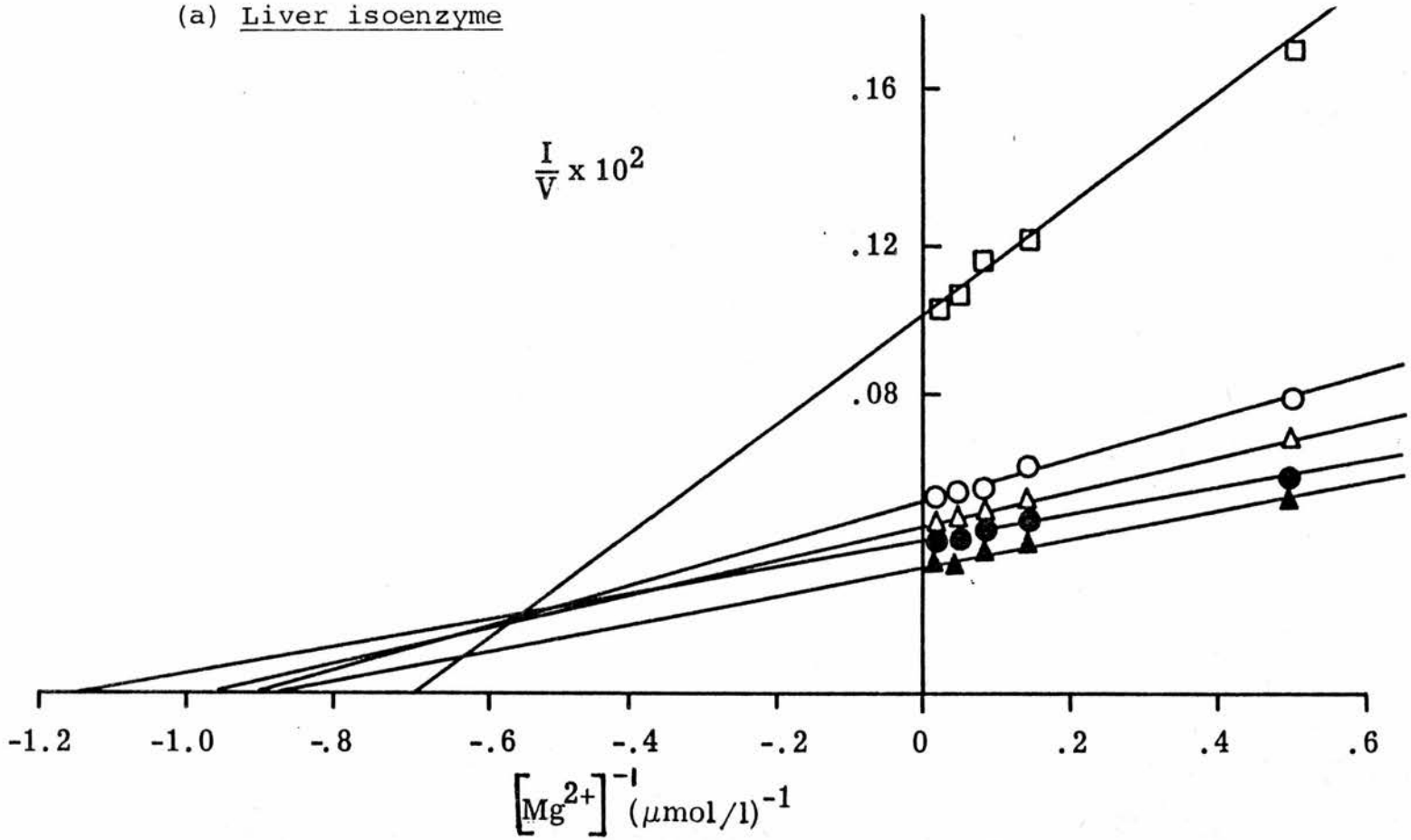


Figure 3.11. (continued).

Figure 3.12. 1/v versus $[Mg^{2+}]^{-1}$ plots at selected concentrations of p-nitrophenyl phosphate, where $[Mg^{2+}]$ is taken as the "best fit" magnesium concentration (see text). V is measured as μmoles p-nitrophenol produced per min per litre. —□— 0.5; —○— 1.5; —△— 2.5; —●— 3.5; —▲— 10.0 mmol/l p-nitrophenyl phosphate.



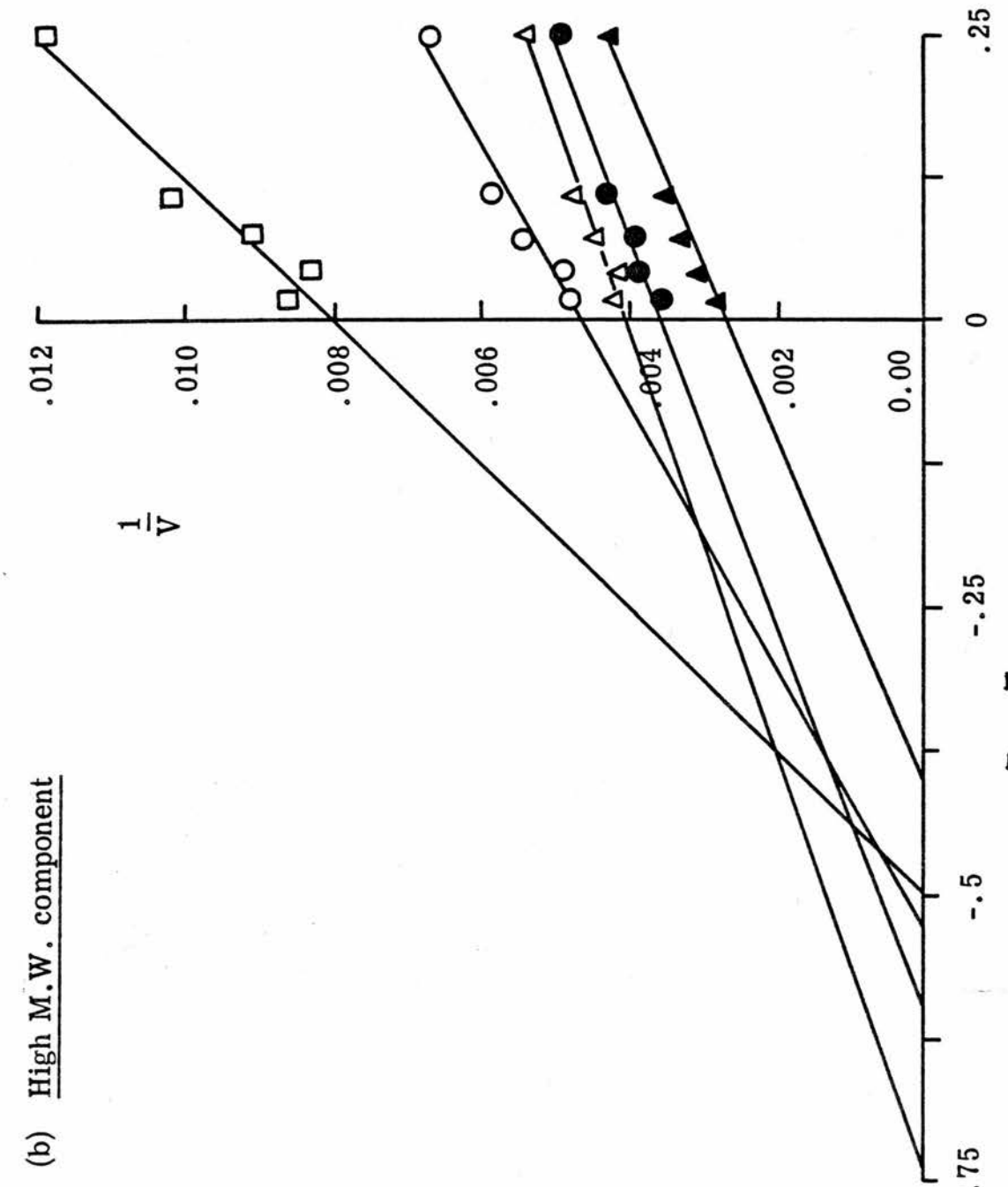


Figure 3.12. (continued).

TABLE 3.8.

Apparent K_As of activation by magnesium ions

Concentration of p-nitrophenyl phosphate (mmol/l)	Apparent K_A (S.E.) (μmol/l)		Apparent V_{max} (S.E.) (iu/l)	
	Liver ALP	High mol wt ALP	Liver ALP	High mol wt ALP
0.5	1.46 (0.08)	2.00 (0.39)	1003 (9)	125 (4)
1.5	1.11 (0.09)	1.88 (0.28)	1982 (23)	215 (5)
2.5	1.04 (0.02)	1.36 (0.18)	2263 (6)	248 (4)
3.5	0.87 (0.10)	1.68 (0.24)	2444 (35)	280 (6)
10	1.14 (0.13)	2.48 (0.42)	3006 (49)	376 (11)

above. For the liver isoenzyme the mean apparent K_A was 1.1 μmol/l and for high mol wt ALP 1.9 μmol/l. No definite conclusions can be drawn concerning the significance of this apparent difference, owing to the assumptions underlying the data used in the calculation.

Some support for the hypothesis that a proportion of the endogenous Mg^{2+} may have been sequestered by protein came from observations on the effect of added Mg^{2+} on the activities of the fractions obtained by Sepharose 6B chromatography. Fig 3.13 shows the ALP elution profiles when the fractions were analysed in the presence and absence of 0.5 mmol/l added magnesium chloride. The ratios of the activities in the presence of added Mg^{2+} to the activities in the absence of added Mg^{2+} were calculated and plotted as an elution profile (Fig 3.14.), omitting those fractions in which activities were negligible. This elution profile varied in parallel with the protein elution profile. The endogenous Mg^{2+} concentration in each fraction, measured by atomic absorption spectrophotometry, varied randomly between 2.0 and 4.0 μmol/l throughout the elution profile. The findings illustrated by Fig 3.14 are therefore best explained by the supposition that some of the endogenous Mg^{2+} was sequestered by protein. This would result in an apparent increase in the activation by added

Figure 3.13. Elution profile of a serum containing liver and high mol wt ALP, following Sepharose 6B chromatography. ALP activity in eluate measured in the presence (—▲—) and absence (—●—) of added 0.5 mmol/l magnesium chloride.

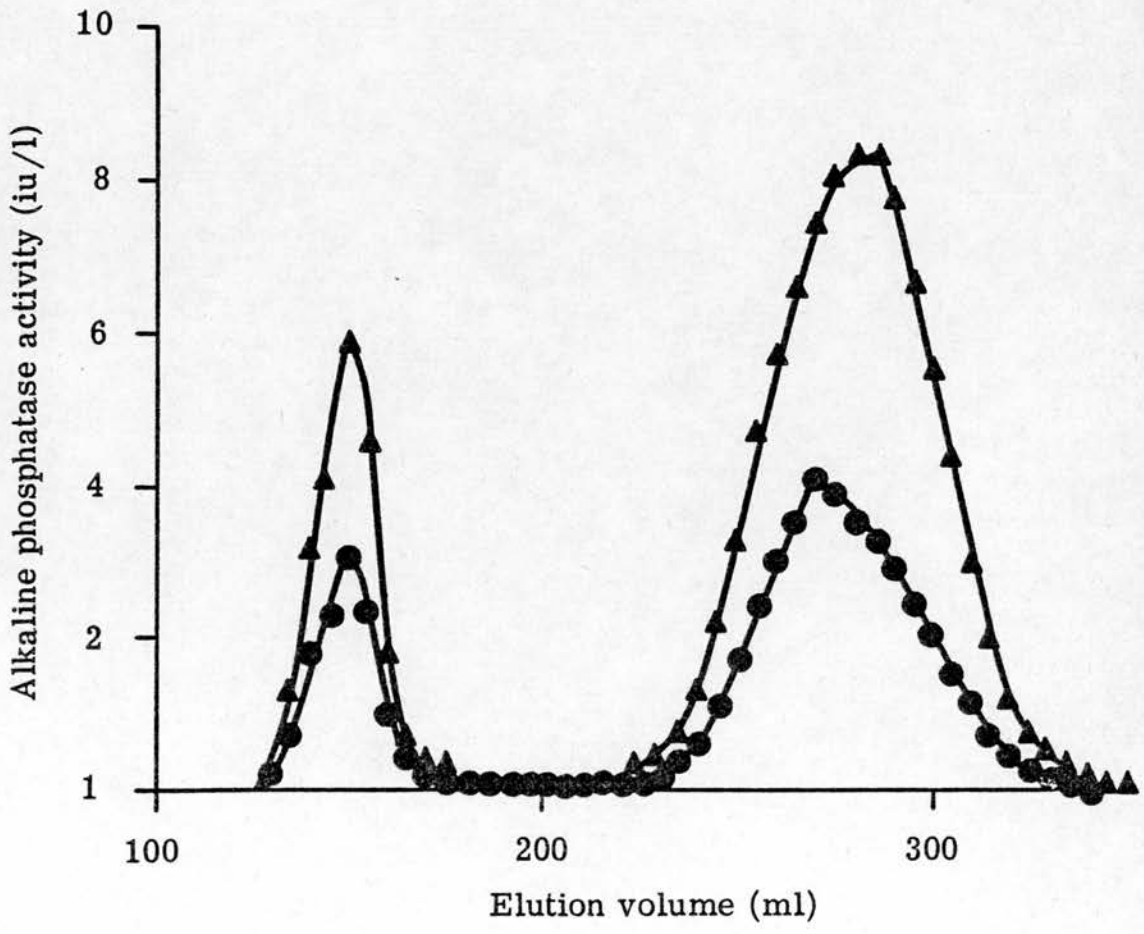
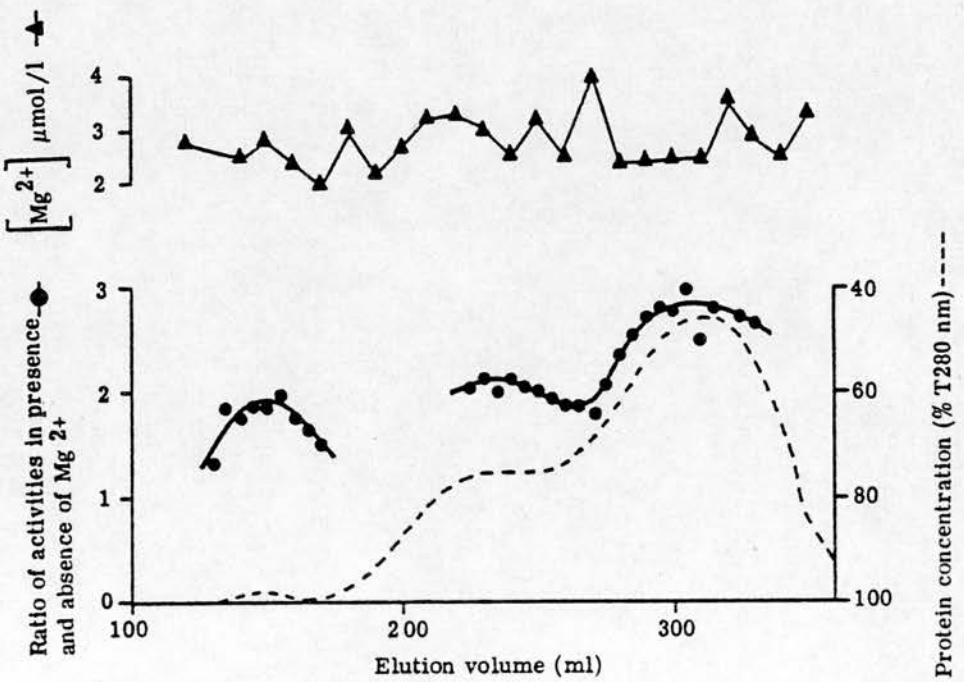


Figure 3.14. Elution profiles of the serum in Fig 3.13 following Sepharose 6B chromatography, showing relationship of activation by magnesium ions to protein elution profile (- - -) - see text for details. —▲— endogenous magnesium concentration in fractions; —●— ratio of ALP activity measured in the presence of 0.5 mmol/l Mg^{2+} to activity measured in the absence of added Mg^{2+}.



Mg^{2+} in parallel with the increase in protein concentration. An attempt to demonstrate this effect by adding 2 g/l human albumin to the reaction buffer was unsuccessful since the albumin preparation contained Mg^{2+}, as measured by atomic absorption spectrophotometry.

3.2.7. Effect of divalent metal ions other than magnesium

The activities of liver and high mol wt serum ALP were measured in the absence and in the presence of 1 μmol/l and 100 μmol/l divalent metal ions. Calcium and nickel ions were present in the form of their chloride salts, manganese and zinc ions in the form of their sulphate salts.

TABLE 3.9.

Inhibition by divalent metal ions

Metal ion	Concentration (μ mol/l)	% inhibition	
		Liver ALP	High mol wt ALP
Calcium	1	9	3
	100	6	3
Manganese	1	7	4
	100	13	11
Nickel	1	0	4
	100	26	16
Zinc	1	6	8
	100	71	65

Table 3.9. shows that 1 μmol/l of all 4 divalent metal ions caused only slight inhibition of the activities of the two

isoenzyme preparations, and to a similar degree in both cases. At 100 μmol/l calcium and manganese ions still only inhibited the isoenzymes slightly. Nickel ions showed a slightly more marked inhibitory effect on both isoenzymes at this concentration. Zinc ions at 100 μmol/l inhibited both isoenzymes markedly.

3.2.8. Summary

High mol wt serum ALP resembled the liver isoenzyme with regard to:

1) buffer specificity
2) pH optima
3) inhibition by L-homoarginine
4) inhibition by divalent metal ions

Both high mol wt and liver ALP were activated by Mg^{2+} but it was not possible to draw conclusions concerning the similarity or otherwise of the apparent K_A's owing to probable ion-binding effects.

However, high mol wt ALP differed from the liver isoenzyme, and also from the bone isoenzyme and high and low mol wt biliary ALP, with regard to its affinities for the substrates α-naphthyl acid phosphate and p-nitrophenyl phosphate.

CHAPTER 4

PHYSICAL AND BIOCHEMICAL CHARACTERISTICS

The large molecular size of high mol wt ALP might result from aggregation of liver isoenzyme molecules or association of ALP with a) protein alone, b) lipid alone, c) carbohydrate alone or d) a combination of these moieties in the form of membrane fragments. Owing to the histological location of ALP on the sinusoidal and biliary canalicular membranes of the hepatocyte and the observations of Shinkai and Akedo (1972) that LAP and 5'NT also occurred in a high mol wt form, it was the latter hypothesis which was principally tested by the experiments presented in this chapter. Following preliminary studies on molecular size, charge and stability to heat and urea, the relationship of high mol wt ALP both in serum and bile to the membrane marker enzymes, γGT, LAP and 5'NT, was therefore investigated. Its relationship to LPX was also studied since this, like high mol wt ALP, has a high mol wt and appears in the serum of patients with cholestasis. Finally, the effects of various agents, which have been used to solubilise membranes, on the structural integrity of high mol wt ALP were explored.

4.1. MOLECULAR WEIGHT

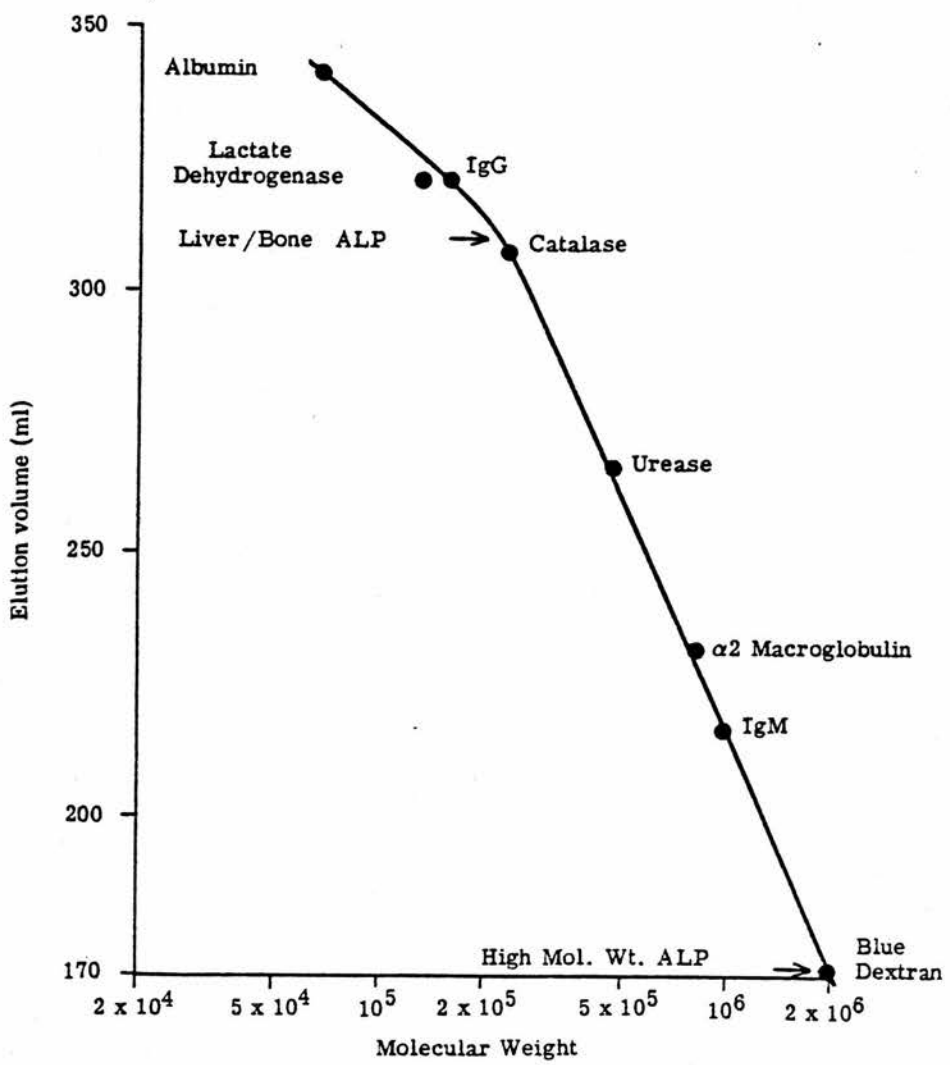
A 95 x 2.5 cm Sepharose 6B column (bed volume: 238 ml; void volume: 171 ml; flow-rate: 28 ml/hour) was calibrated by proteins of mol wts ranging from 6.9×10^4 to 1.0×10^6 as shown in Table 4.1. The semi-logarithmic calibration curve for the column (Fig 4.1.) is linear between mol wts 2.5×10^5 and 1.0×10^6 but tails off in the lower mol wt range below 2×10^5.

TABLE 4.1.

Proteins used in the mol wt calibration of a Sepharose 6B column

Protein	Mol wt	Method used for determination
IgM	1.0×10^6	Radial immunodiffusion (Mancini et al, 1965)
α_2 macroglobulin	8.2×10^5	Radial immunodiffusion (Mancini et al, 1965)
Urease	4.8×10^5	Urea substrate: ammonium ion estimated by Nessler's reagent (Gentzkow, 1942)
Catalase	2.4×10^5	Hydrogen peroxide substrate; reaction rate at 240 nm (Beers and Sizer, 1952)
IgG	1.6×10^5	Radial immunodiffusion (Mancini et al, 1965)
Lactate dehydrogenase	1.4×10^5	Pyruvate/NADH substrates; reaction rate at 340 nm (Scand. Soc. Clin. Chem. Clin. Physiol. recommended method, 1974)
Albumin	6.9×10^4	Absorption at 280 nm

Figure 4.1. Semi-logarithmic plot of elution volume versus mol wt in the calibration of a 95 x 2.5 cm Sepharose 6B column.



Liver and bone ALP were found to have similar mol wts of 2.2×10^5. High mol wt ALPs from both serum and bile were excluded from the gel matrix and appeared in the void volume. It was concluded that their mol wts exceeded 1×10^6. Since no reliable mol wt markers are available above this mol wt, it was not considered worthwhile to proceed to a larger mesh gel e.g. Sepharose 2B.

In order to check these findings, various isoenzymes of ALP, in purified form and in the native serum or bile, were subjected to electrophoresis to equilibrium in 4 to 26% polyacrylamide gradient gels. Serum proteins acted as mol wt markers (Fig 4.2.). This procedure confirmed the mol wts found by gel filtration (Table 4.2.). The high mol wt

TABLE 4.2.

Mol wts of various ALP isoenzymes, determined by electrophoresis to equilibrium in polyacrylamide gradient gel

ALP isoenzyme		Mol wt
Liver	from serum	2.2×10^5
Bone	from serum	2.2×10^5
Intestine	from serum	1.95×10^5
High mol wt	from serum	$> 1 \times 10^6$
Low mol wt	from bile	2.2×10^5
High mol wt	from bile	$>1 \times 10^6$

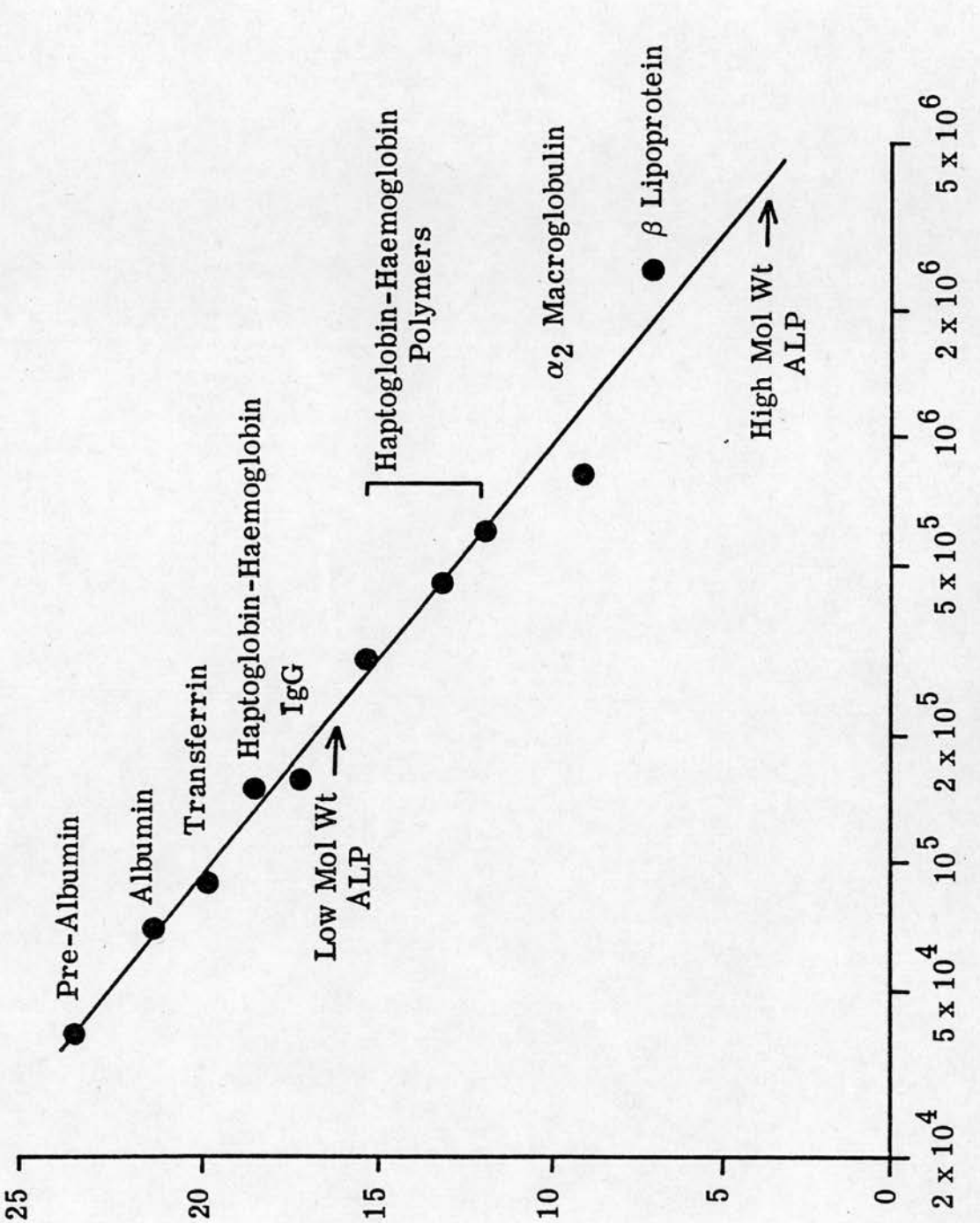


Figure 4.2. Semi-logarithmic plot of polyacrylamide gel concentration versus mol wt in the calibration of a 4 to 26% polyacrylamide gradient gel.

components from both serum and bile migrated exactly the same distance into the gel. In addition, the low mol wt isoenzyme in bile was found to have a mol wt similar to that of the liver isoenzyme (2.2×10^5).

The estimate of 2.2×10^5 for the mol wt of the liver isoenzyme is in close agreement with previous measurements made in the presence of NaCl but at variance with measurements made in the absence of NaCl which indicated a mol wt of 1.46×10^5 (Trépanier et al, 1976). This may be due to non-specific adsorption of the glycoprotein to the Sephadex, Sepharose or polyacrylamide gel matrix in the absence of NaCl. On the other hand, in the presence of NaCl there may be some unfolding of the polypeptide chains leading to an alteration in the Stokes radius (c.f. Ca^{2+}-activated adenosine triphosphatase, Farias et al, 1975). The large size of the high mol wt ALP is also in agreement with previous studies which have shown it to be excluded not only by Sephadex G200 but by Sepharose gels 6B, 4B and 2B in order of increasing mesh size (Akedo et al, 1967; de Broe et al, 1975).

4.2. CHARGE

The high mol wt ALPs from both serum and bile migrated in the α_1 globulin position (ahead of the normal liver isoenzyme and of all the other ALP isoenzymes which

normally occur in serum) during electrophoresis in cellulose acetate, which is the only electrophoretic medium to exert no molecular sieving effect (section 2.5, Fig 2.2.). Both high mol wt ALPs also eluted from an ion-exchange column at a higher ionic strength (135 mmol/l) than all other commonly encountered isoenzymes of ALP (80 mmol/l) (section 3.1.2., Fig 3.1.). These ionic strengths are in agreement with those observed for biliary ALP by Price et al (1972). The high mol wt ALPs from serum and bile must therefore carry an identical net negative charge which is higher than any other commonly occurring ALP isoenzyme at alkaline pH.

4.3. EFFECT OF NEURAMINIDASE

Sera and purified ALP isoenzymes were incubated for 2 hours at 37°C with 100 iu/l neuraminidase in 0.05 mol/l acetate buffer pH 5.5 containing 0.15 mol/l NaCl and 9 mmol/l $CaCl_2$. The incubation mixture contained 2 volumes serum/enzyme preparation to 1 volume neuraminidase solution (Fishman, Inglis and Ghosh, 1968).

Electrophoresis in 2.5% polyacrylamide gel demonstrated that the neuraminidase-treated liver and bone ALP migrated more slowly than the corresponding untreated specimens. On the other hand, the migration rates of the neuraminidase-treated intestinal and high mol wt ALP were not affected. Since it was possible that the molecular

sieving effect of the gel on the high mol wt ALP might be masking a real alteration in charge, electrophoresis was repeated in cellulose acetate. The results (Table 4.3.) showed that the migration rates of both liver and high mol wt ALP were retarded to similar extents by neuraminidase treatment in this medium. An interesting observation was

TABLE 4.3.

Reduction in electrophoretic mobility of liver and high mol wt ALP by the action of neuraminidase. The electrophoretic medium was cellulose acetate

ALP Isoenzyme		Percentage retardation by neuraminidase
Liver	in whole serum	20
High mol wt		23
Liver	purified preparations	75
High mol wt		61

that the retardation was much greater for both isoenzymes in the purified state than it was in whole serum. This may have been due to competition for the active site of neuraminidase from other sialic acid-containing proteins in serum, or because in serum the sialic acid residues were somehow more protected. The effect of neuraminidase on the high mol wt ALP in bile was not studied but it seems likely

that the high negative charge of high mol wt ALP in both serum and bile is largely caused by the presence of the sialic acid residues.

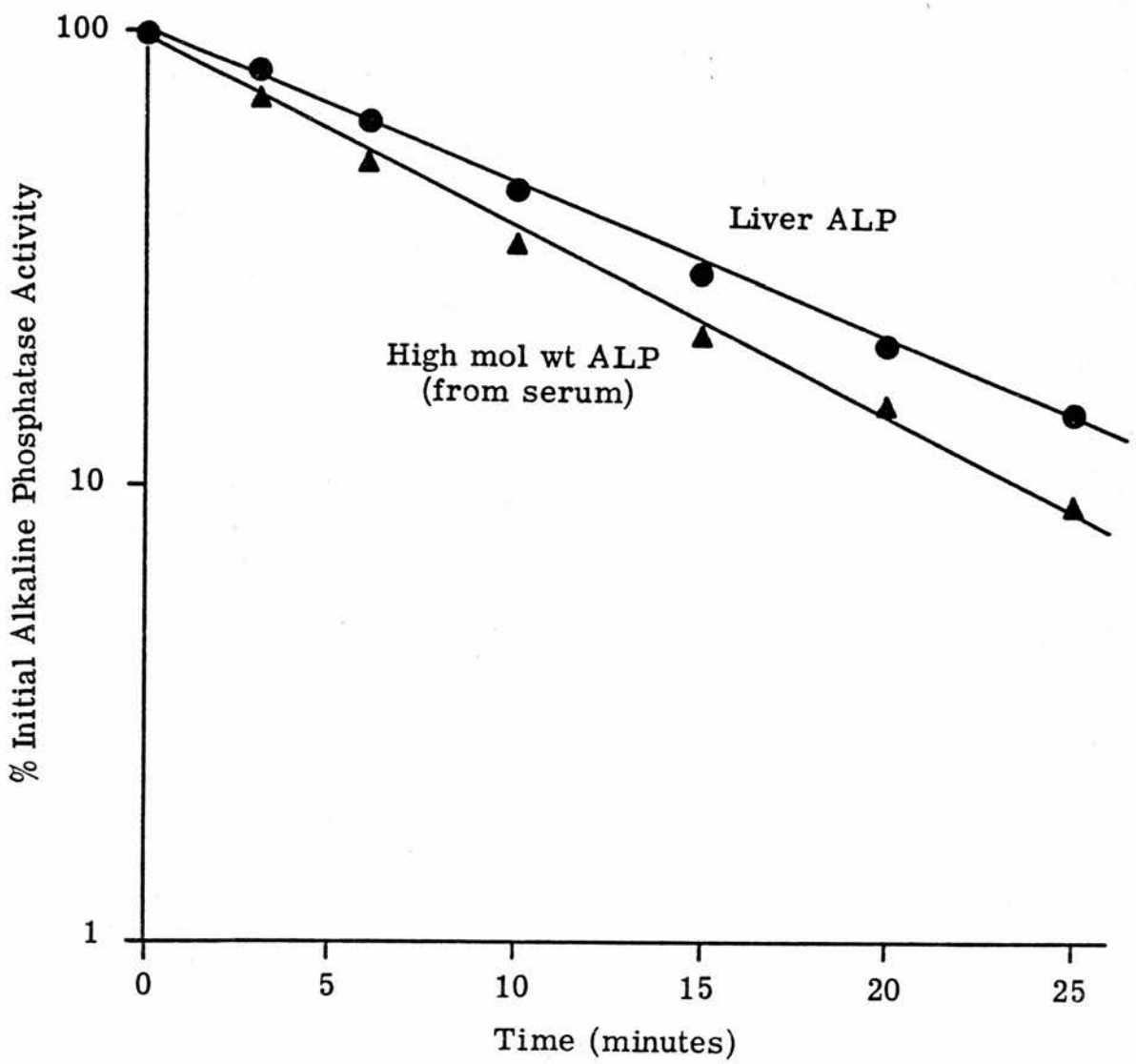
4.4. INACTIVATION BY UREA

Urea is a highly polar molecule which acts by disrupting hydrogen bonds. There are two types of inhibition of ALP by urea: the first, at concentrations below 3 mol/l, is reversible, instantaneous and non-competitive in nature; the second, at concentrations above 3 mol/l, is irreversible and time-dependent (Birkett et al, 1967). It was the second type of inhibition that was investigated here.

At time zero, partially purified preparations of liver and high mol wt ALP were mixed with equal volumes of urea solution to a final concentration of 5 mol/l urea. The mixtures were incubated at room temperature. At timed intervals, 50 µl samples were withdrawn, mixed immediately with 1 ml 0.9 mol/l diethanolamine buffer pH 10.3 containing 0.5 mmol/l $MgCl_2$ (to dilute out the urea and prevent further inactivation), and the ALP activities measured.

The inactivation of both liver and high mol wt ALP (from serum) followed mono-exponential decay curves up to 25 minutes (Fig 4.3.) with half-lives of 9.0 min and 7.0 min respectively, estimated graphically. After 25 minutes

Figure 4.3. Semi-logarithmic plot of ALP inactivation by 5 mol/l urea versus time.



the rate of decay of both isoenzymes diminished.

Electrophoresis of the urea-treated samples in 2.5% polyacrylamide gel showed diminished staining intensity but no qualitative alteration in the positions of the isoenzyme bands. Urea, which interferes with hydrogen bonding, therefore probably caused unfolding of the ALP polypeptide chains to a similar extent in liver and high mol wt ALP without disrupting the link between ALP and the other moieties present in high mol wt ALP.

4.5. INACTIVATION BY HEAT AT 56°C

In a two component system in which both the components show exponential decay when heated,

$$\text{remaining activity} = Ae^{-k_1 t} + Be^{-k_2 t}$$

where t is the time of exposure to heat, A and B are the percentage activities of each component and k_1, k_2 are decay constants appropriate to each component. Using this formula and a curve fitting program on a Hewlett-Packard desk-top computer a bi-exponential decay curve could be fitted to a series of experimental points assuming constant variance for all points. The same program was used to calculate the percentage and half-life of each component in the system.

Partially purified preparations of ALP were heated at 56°C ($\pm$ 0.5°C) for varying lengths of time according to

the method of Whitby and Moss (1975). The inactivation curves obtained were mono-exponential for the liver and bone isoenzymes but bi-exponential for high mol wt ALP from serum and bile (Fig 4.4.), the two components being present in approximately equal amounts in each case (Table 4.4.). One component in each case was heat labile whereas the second component was much more heat-stable (Table 4.4.). Overall, high mol wt ALP from both serum and bile was slightly more heat-labile than the liver isoenzyme.

In whole serum, it is doubtful whether the slight difference in heat lability between liver and high mol wt ALP could be resolved into two separate components. Therefore the contribution by high mol wt ALP to total serum ALP in different proportions in liver disease may partly explain the observed variation in apparent liver isoenzyme half-life from serum to serum (Whitby and Moss, 1975).

The two components present in high mol wt ALP could not be resolved by Sepharose 6B or ion-exchange chromatography. The apparent heterogeneity may have existed in the original serum and bile; alternatively, it may have been an artefact arising from the purification procedure. Possible explanations for these findings are discussed

Figure 4.4. Semi-logarithmic plot of ALP inactivation by heat at 56°C versus time. -▲- liver isoenzyme; -●- bone isoenzyme; -△-high mol wt ALP (from serum; -·o-·high mol wt ALP (from bile).

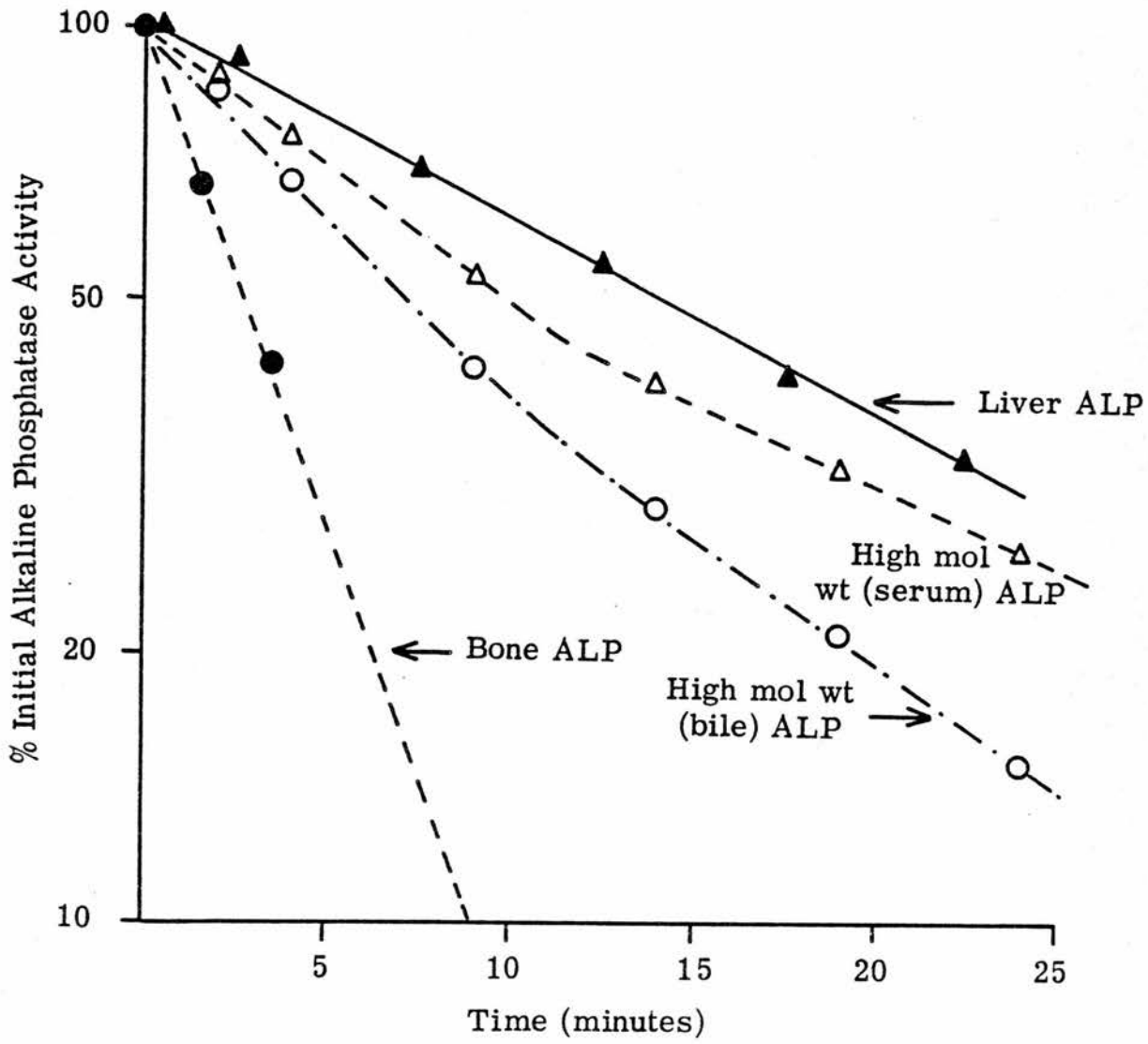


TABLE 4.4.

The half-lives at 56°C of the liver and bone isoenzymes and of high mol wt ALP purified from serum and bile; and the percentage of each component in the biphasic exponential decay curves

ALP isoenzyme	Percentage of component [S.E.]		Half-life of component (minutes) [95% confidence limits]*	
	Heat-labile	Heat-stable	Heat-labile	Heat-stable
Liver +	-	100	-	14.0
Bone + from serum	100	-	3.8	-
High mol wt	54[4]	46[5]	4.7[3.9-5.7]	22.4[18.5-28.2]
High mol wt from bile	49[4]	51[6]	2.6[1.9-4.0]	11.6 [9.8-14.4]

* Half-lives and confidence limits derived from $k \pm 2$ S.E.

\+ Curves monoexponential. Half-lives estimated graphically, therefore 95% confidence limits not calculated.

more fully in Chapter 7.

4.6. RELATIONSHIP OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE TO HIGH MOLECULAR WEIGHT COMPONENTS OF VARIOUS MEMBRANE MARKER ENZYMES

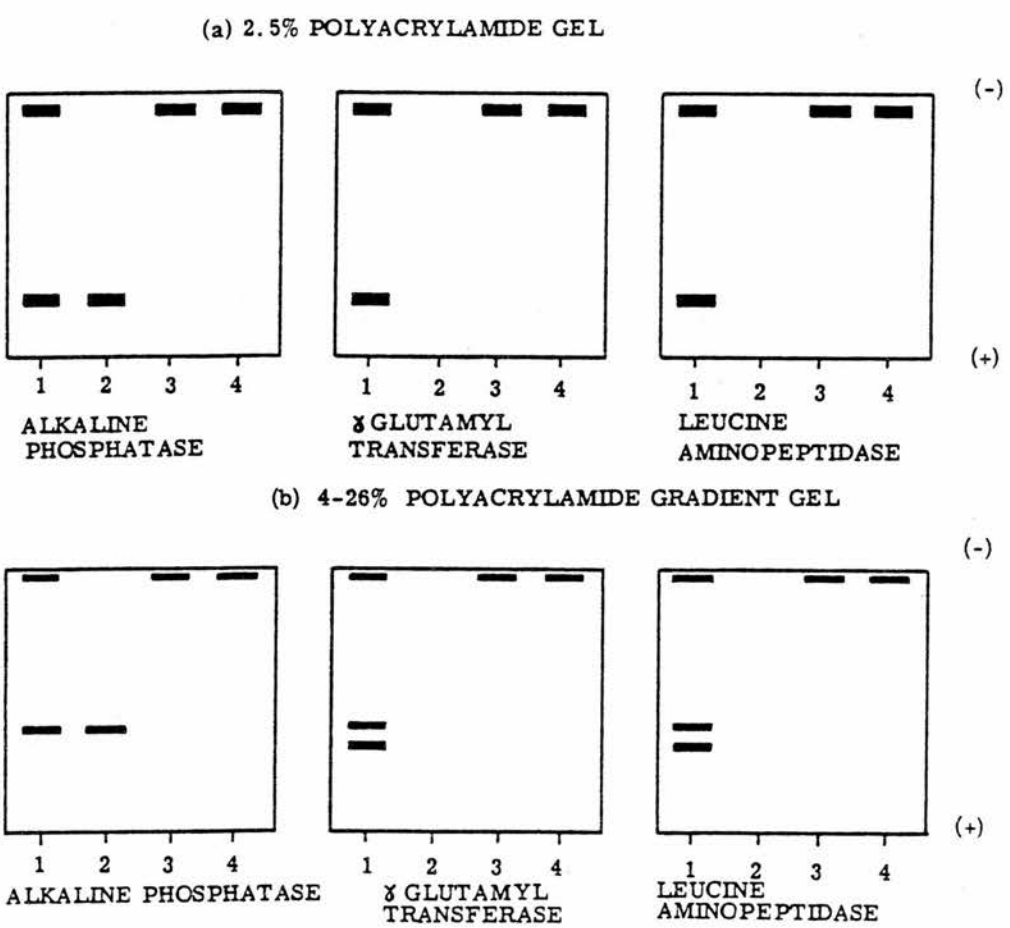
Various enzymes are known to be located principally on the plasma membrane of the hepatocyte. Besides ALP itself, these include γGT, LAP and 5'NT. The relationship between these enzymes was explored in a number of ways.

4.6.1. Electrophoresis

Purified preparations. Electrophoresis of purified high mol wt ALP from serum and bile in 2.5% polyacrylamide gel gave a single band of ALP activity near the origin. A single band in exactly the same position was found when the gel was stained for γGT and LAP. Similarly, electrophoresis to equilibrium in a 4 to 26% polyacrylamide gradient gel gave a single band of activity near the origin for all 3 enzymes (Fig 4.5.). This showed that the high mol wt components of all 3 enzymes had a similar mol wt.

Sera. 9 sera which contained high mol wt ALP (demonstrated by electrophoresis) invariably also contained high mol wt γGT and LAP. Conversely, 9 sera which did not contain high mol wt ALP did not have high mol wt γGT or LAP. In

Figure 4.5. Positions of ALP, ɣGT and LAP isoenzymes following electrophoresis in a) 2.5% polyacrylamide gel and b) 4 to 26% polyacrylamide gel (to equilibrium). Samples: 1, serum containing liver and high mol wt ALP; 2, purified liver ALP; 3, purified high mol wt ALP from serum; 4, purified high mol wt ALP from bile.



other words, these high mol wt enzymes appeared to be present or absent in unison.

4.6.2. Sepharose 6B chromatography

Serum. 7 sera containing varying amounts of liver and high mol wt ALP were eluted from a 95 x 2.6 cm Sepharose 6B column. The original sera and the eluted fractions were assayed for the membrane marker enzymes ALP (6 sera), γGT (7 sera), LAP (7 sera) and 5'NT (5 sera). The elution profile of one of these sera is shown as an example in Fig 4.6. In addition to various low mol wt isoenzymes, all four enzymes possessed a high mol wt component which always eluted in the void volume. By contrast, lactate dehydrogenase (a cytoplasmic enzyme) did not have a high mol wt component in serum.

The total activities of enzyme present in the high and low mol wt peaks were calculated and the high mol wt component in the original serum was expressed either as iu/l or as a percentage of total enzyme activity. The total activities of the enzymes were significantly correlated with one another (Fig 4.7., Table 4.5.(a)). Similar significant correlations were observed between the high mol wt enzymes, expressed as iu/l (Fig 4.8., Table 4.5.(b)). Much lower correlations occurred between these high mol wt enzymes when they were expressed merely as a

Figure 4.6. Elution profiles of ALP, ɣGT, LAP and 5'NT activities on a Sepharose 6B column following application of serum from a patient with liver disease.

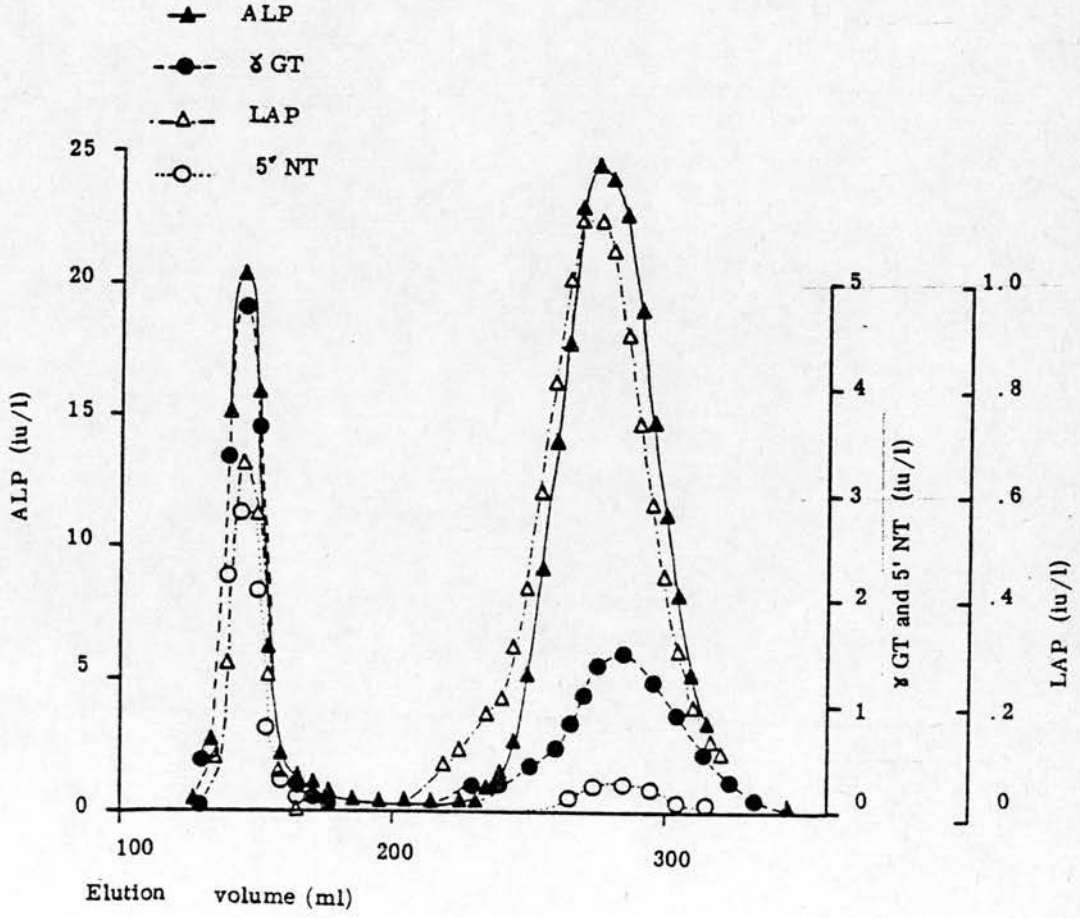


Figure 4.7. Total activities of ALP, ɣGT, LAP and 5'NT plotted against one another in pairs (7 sera). The arrow indicates serum from a patient with alcoholism.

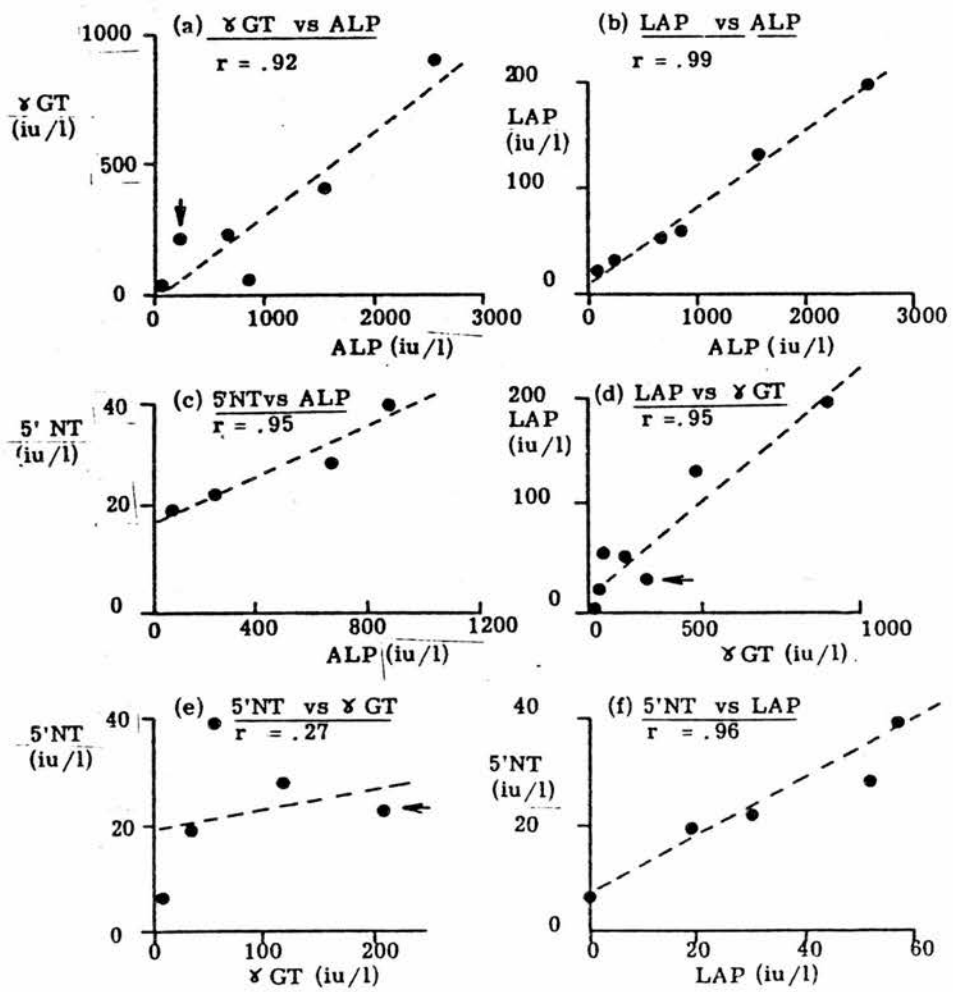


Figure 4.8. High mol wt component activities of ALP, γGT, LAP and 5'NT plotted against one another in pairs (7 sera). The arrow indicates serum from a patient with alcoholism.

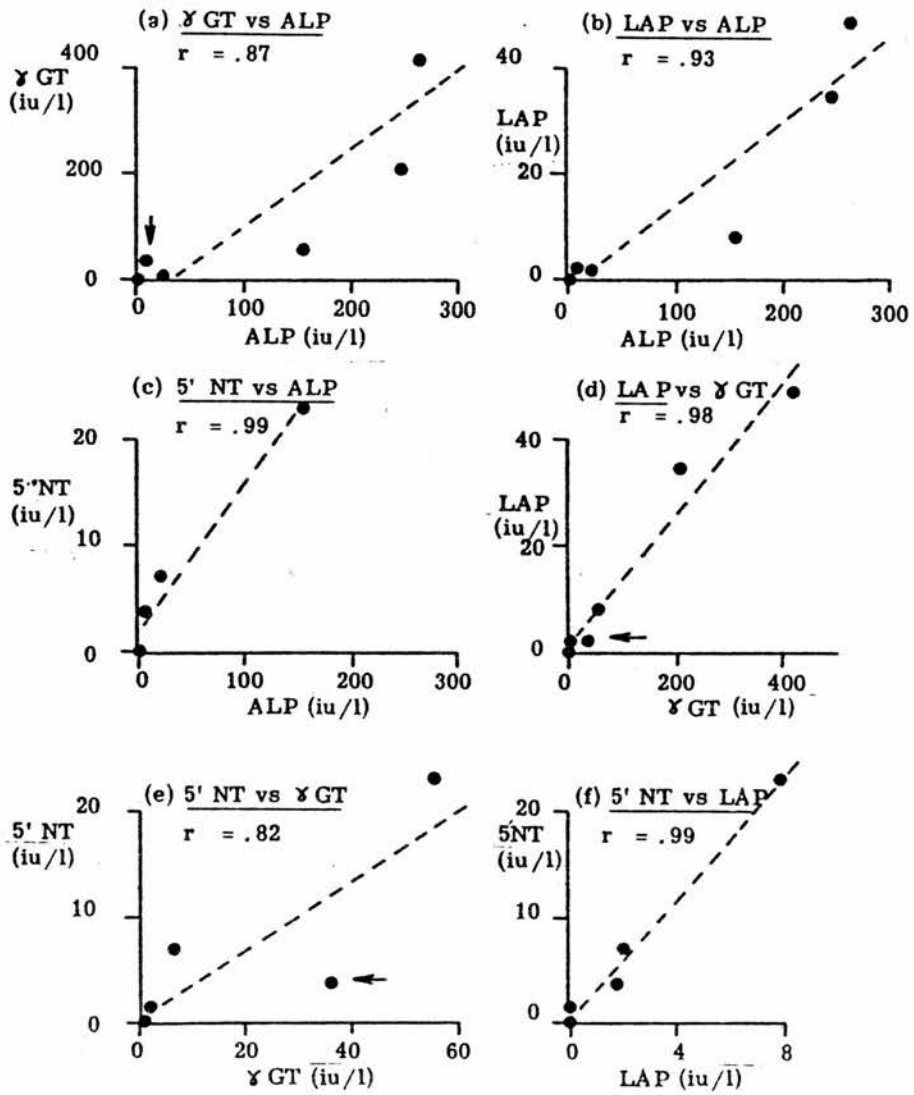


TABLE 4.5.

Two-tailed probabilities associated with the correlation coefficients between pairs of enzymes

(a) Total enzyme activities (iu/l)

	ALP	γGT	LAP	5'NT
ALP		.05	.001	.05
γGT			.01	N.S.
LAP				.01
5'NT				

(b) High mol wt forms (iu/l)

	ALP	γGT	LAP	5'NT
ALP		.05	.01	.05
γGT			.001	N.S.
LAP				.001
5'NT				

(c) High mol wt forms (percentage of total activity)

	ALP	γGT	LAP	5'NT
ALP		.05	N.S.	.05
γGT			N.S.	N.S.
LAP				.05
5'NT				

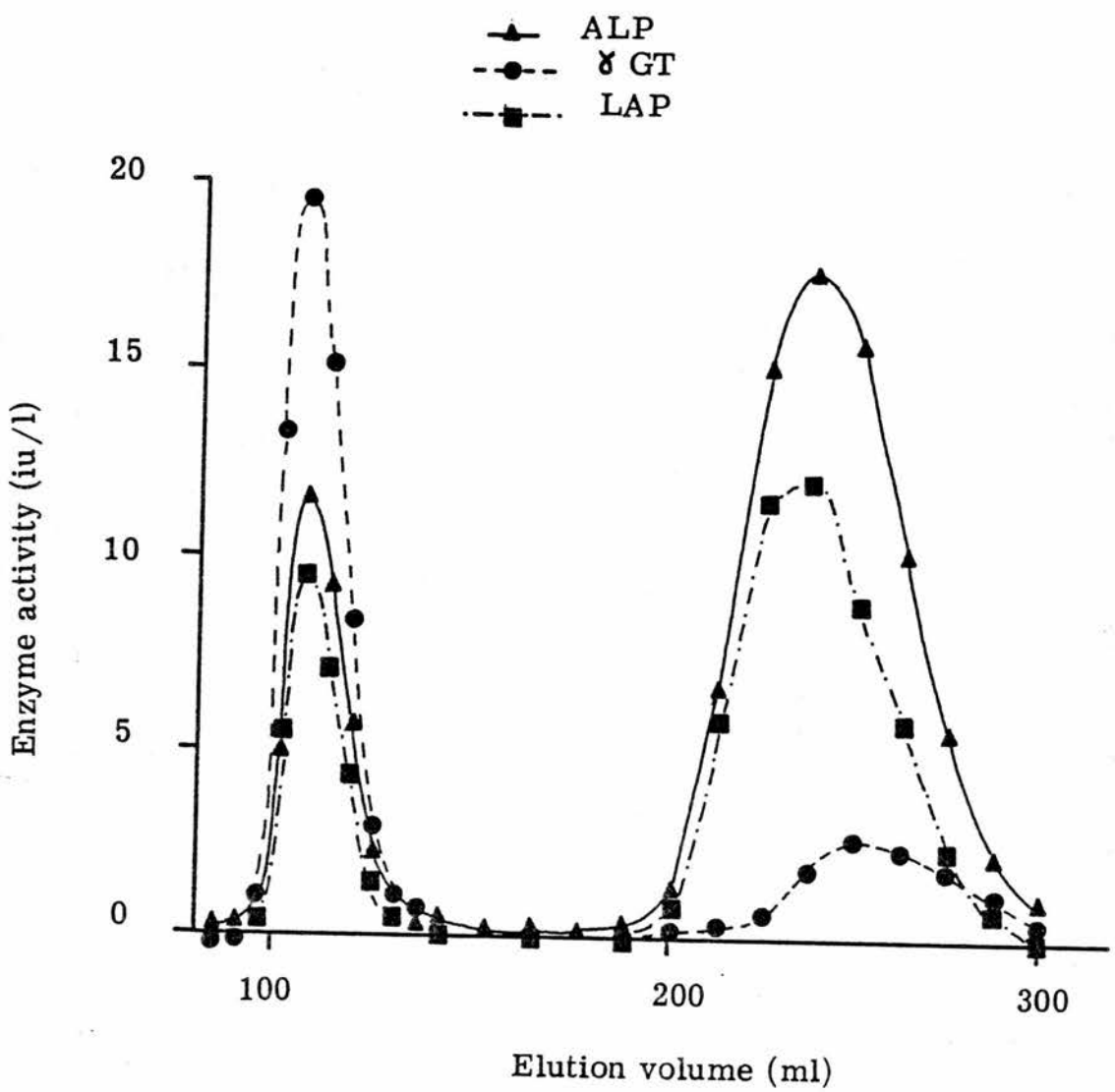
percentage of the total activity (Table 4.5.(c)). It seems likely, therefore, that the high mol wt enzymes are correlated with one another in their own right, and not just because the total activities are correlated. Whether they always vary in parallel, indicating a common control mechanism, or whether certain factors may influence the activity of one high mol wt enzyme to a greater extent than the others could be discovered by studying further patients. In Figs 4.7. and 4.8. the arrow indicates a patient with chronic alcoholism, in whom γGT activity may therefore have been induced, but this case had a variable effect on the correlation coefficients.

Bile. A concentrated sample of normal hepatic bile, from which most of the lipid and pigment had been removed (section 3.1.1.), was also eluted from the Sepharose 6B column. In addition to various low mol wt isoenzymes, ALP, γGT and LAP all showed prominent high mol wt peaks in the void volume (Fig 4.9.). This suggests that any qualitative relationship between the high mol wt components of the four enzymes in serum may also be true of their high mol wt components in bile.

4.6.3. Ion-exchange chromatography

Serum containing liver and high mol wt ALP was dialysed overnight against 0.01 mol/l Tris-HCl buffer pH

Figure 4.9. Elution profiles of ALP, ɣGT and LAP activities on a Sepharose 6B column following application of concentrated normal hepatic bile from which lipid and pigment have been removed.



7.5. 5 ml of this dialysed serum was applied to a 16 x 2.5 cm column of DEAE-cellulose pre-equilibrated with the same buffer, and subjected to salt (NaCl) gradient chromatography. The elution profiles of ALP, γGT and LAP each showed two distinct peaks, although these did overlap using this gradient (Fig 4.10). None of these peaks coincided in the ionic strengths at which they eluted.

In order to identify the enzyme peaks, four pools, A, B, C, and D were made as shown in Fig. 4.10. These were concentrated approximately 10-fold and applied consecutively to a Sepharose 6B column. ALP, γGT and LAP activities were measured in the eluate (Fig. 4.11.). Each pool was also subjected in triplicate to electrophoresis to equilibrium in 4 to 26% polyacrylamide gradient gel, followed by staining for the three enzymes.

The results (summarised in Tables 4.6 and 4.7) show that for each enzyme, the first peak to appear on ion-exchange chromatography corresponded to the low mol wt component(s) and the second peak to the high mol wt component. Therefore the high mol wt enzyme peaks did not coincide exactly in their elution positions but partially overlapped. There are various possible explanations for the partially overlapping peaks of the high mol wt enzymes. Firstly, dissociation of a single complex containing all 3 enzymes

Figure 4.10. Elution profiles of ALP(—●—), γ GT (-▲-) and LAP (-·■-·) from a DEAE-cellulose ion-exchange column following application of a serum from a patient with liver disease. —o— ionic strength gradient.

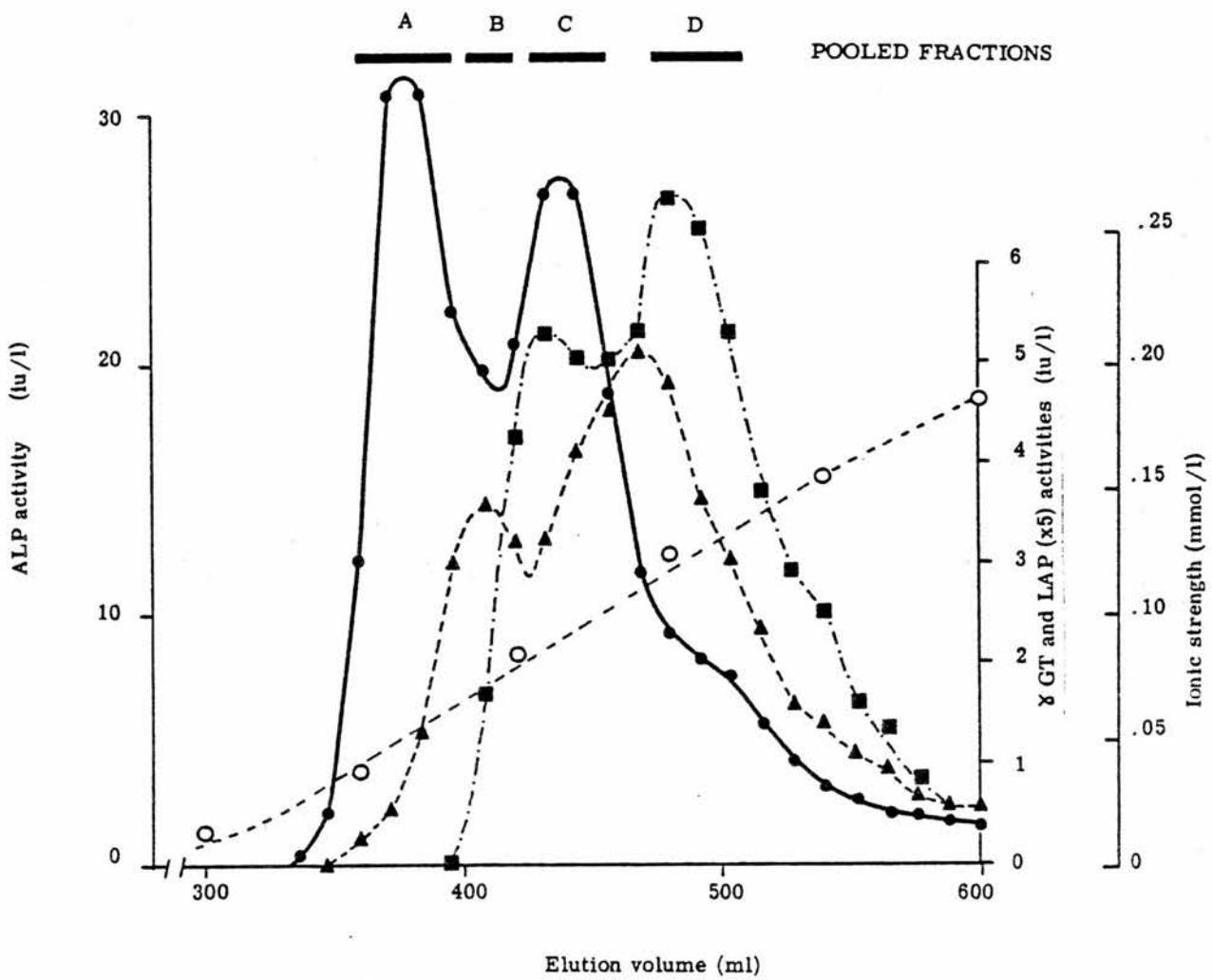


Figure 4.11. Elution profiles of a) ALP b) ɣGT and c) LAP from a Sepharose 6B column following application of pooled fractions A, B, C and D respectively from the DEAE-cellulose ion-exchange column.

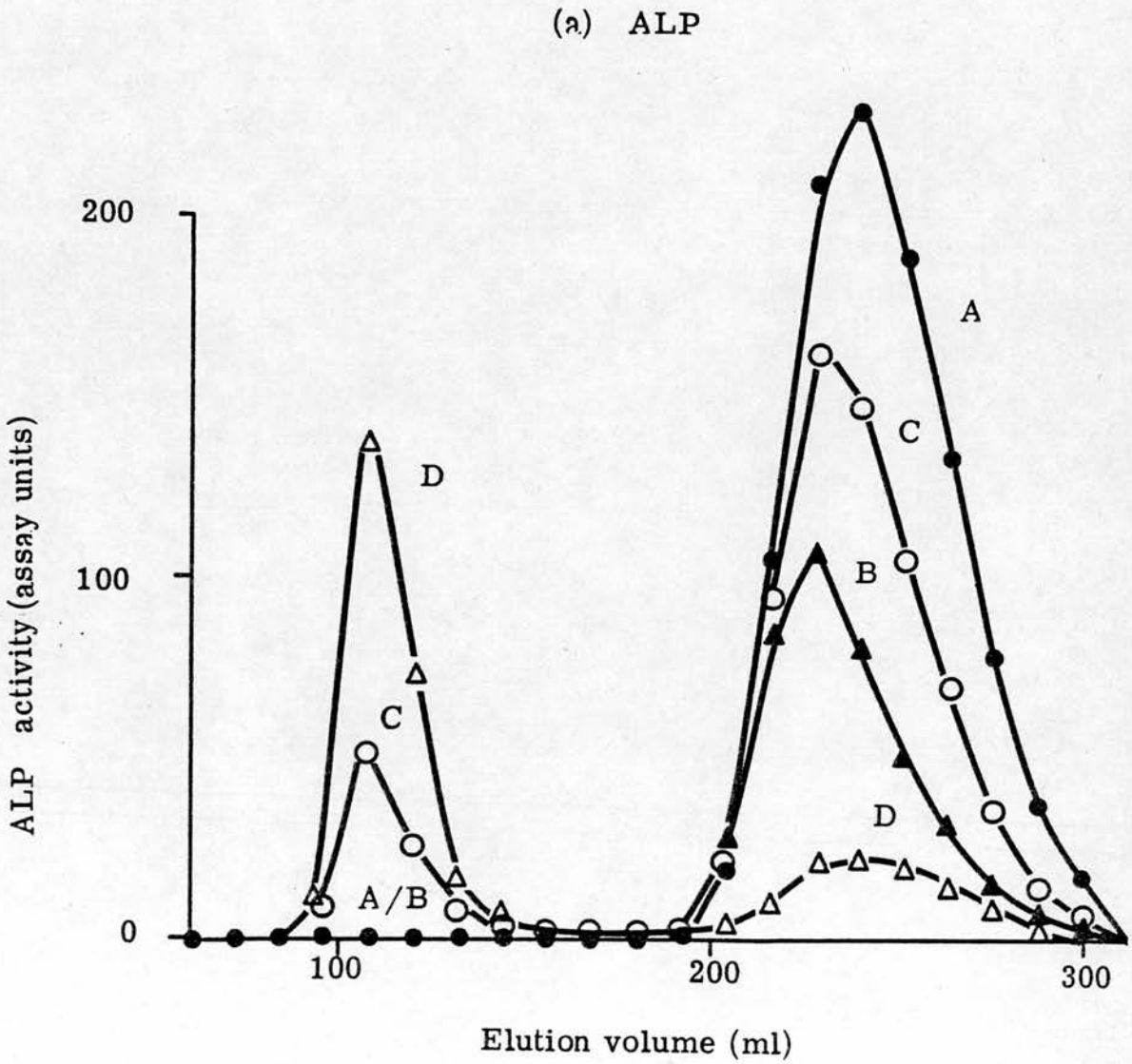


Figure 4.11. (continued)

(b) ɣ GT

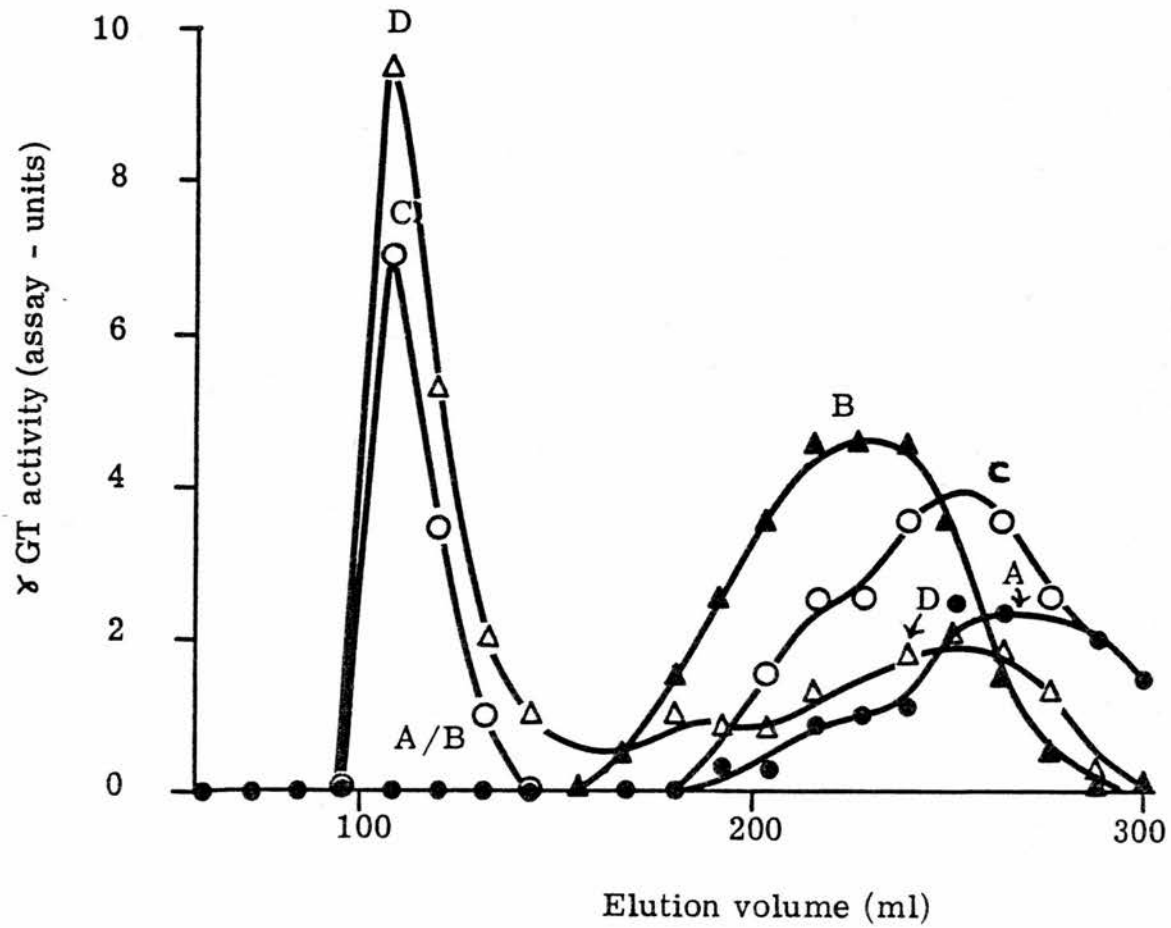


Figure 4.11. (continued)

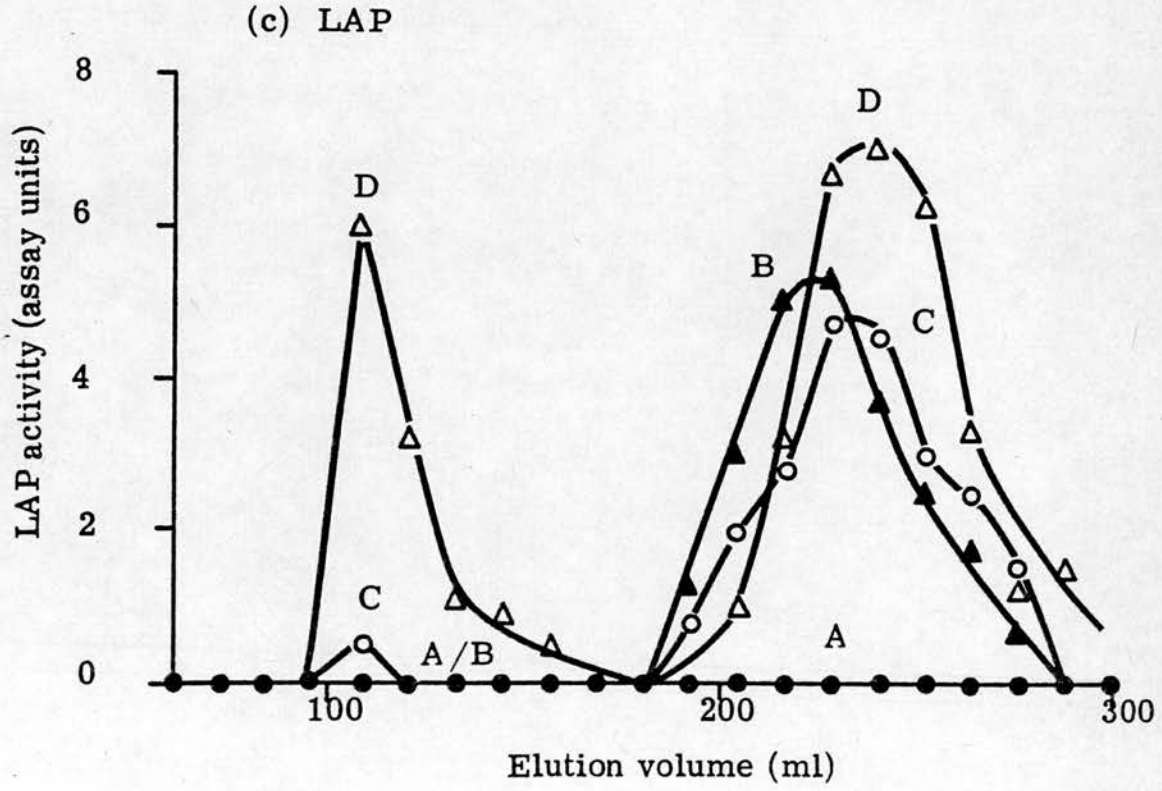


TABLE 4.6.

Summary of ion-exchange and Sepharose 6B peaks and electrophoretic isoenzyme bands present in each of four sets of pooled fractions following ion-exchange chromatography of a single serum. See text for details

Pool	Ion-exchange peaks present			Sepharose 6B chromatography % high mol wt component			Gradient gel electrophoresis		
	ALP	γGT	LAP	ALP	γGT	LAP *	ALP	γGT	LAP
A	1	1	-	0	0	0	Liver	Low mol wt	None
B	1 and 2	1	1	5.3	0	0	Liver	Low mol wt	Low mol wt
C	1 and 2	1 and 2	1 and 2	12.7	33.3	2.2	Liver/ High mol wt	Low/ High mol wt	Low/ High mol wt
D	largely 2	largely 2	largely 2	68.2	60.9	25.4			

* LAP ion-exchange peak 2 appears to contain a low mol wt isoenzyme in addition to the high mol wt component.

TABLE 4.7.

Ionic strengths at which low and high mol wt ALP, γGT and LAP eluted following DEAE-cellulose chromatography of a single serum

Enzyme	Ionic strength (mmol/l) of peak	
	Low mol wt	High mol wt
ALP	64	100
γGT	82	118
LAP	98	126

on the column must be considered. This is unlikely because both before and after ion exchange chromatography the high mol wt enzymes retained their properties, in particular their behaviour on electrophoresis and Sepharose 6B chromatography. Secondly, there may be a series of complexes, each containing only one enzyme. Thirdly, there may be a heterogeneous collection of complexes, all containing all three enzymes in different relative proportions. This would result in the observed pattern of partially overlapping peaks. This is discussed further in Chapter 7.

4.7. EFFECT OF ORGANIC SOLVENTS AND DETERGENTS

4.7.1. Extraction with butan-1-ol

Butan-1-ol (n-butanol) is a solvent which dissolves fats and lipids. It has been extensively used in the solubilisation of ALP from tissues prior to the purification of the enzyme (Morton, 1954). If high mol wt ALP consisted of a complex of ALP with either lipid, lipoprotein or membrane fragments, it might be susceptible to extraction by butanol. If, on the other hand, it consisted of aggregates of ALP or ALP complexed to protein or carbohydrate it would remain unaffected by butanol extraction.

Serum. 5 sera containing liver and high mol wt ALP were mixed with butanol in the proportions of 5 volumes of serum to 1 volume of butanol. Extraction proceeded at 4°C for 30 min with frequent mixing. After centrifugation, the upper fatty butanol layer (which had no detectable enzyme activity) was discarded. Electrophoresis of treated and untreated sera in 2.5% polyacrylamide gel showed that butanol had completely abolished the band corresponding to high mol wt ALP. This was accompanied by a variable increase in the staining intensity of the liver isoenzyme band (Table 4.8.). This is insufficient evidence from which to conclude that butanol actually converts high mol wt ALP into the liver isoenzyme. To

TABLE 4.8.

Effect of butanol extraction on liver and high mol wt ALP in 5 sera, visualised by electrophoresis in 2.5% polyacrylamide gel and quantitated by scanning

Serum	Band staining (experimental units)			
	High mol wt		Liver	
	Untreated	Treated	Untreated	Treated
1	Faint	0	112	137
2	Faint	0	236	212
3	10	0	49	91
4	Faint	0	40	53
5	19	0	129	157

test this hypothesis, studies on separate preparations of liver and high mol wt ALP were required.

Separate preparations of low and high mol wt ALP, γGT and LAP. A similar butanol extraction experiment was conducted on high and low mol wt ALP separated by Sepharose 6B chromatography. The high mol wt component contained ALP, γGT and LAP activity. The low mol wt component, because it was relatively impure, also contained ALP, γGT and LAP activity making it preferable to a purer preparation for the purposes of this experiment.

Electrophoresis of the butanol-treated and untreated preparations, before and after dialysis to remove any remaining butanol, showed that qualitatively

liver ALP was unaffected by treatment with butanol but the band corresponding to the high mol wt component disappeared entirely with no reappearance of activity elsewhere (Fig 4.12.). Removal of the remaining butanol by dialysis did not result in any reappearance of the high mol wt component.

Measurement of enzyme activities demonstrated that high mol wt ALP almost completely and high mol wt γGT and LAP completely disappeared from the aqueous layer after treatment with butanol (Table 4.9.). Nor did the organic layer of butanol show any activity. Dialysis of the aqueous layers did not restore activity. Low mol wt liver ALP showed a slight enhancement of activity when treated with butanol, but the activity of low mol wt γGT was halved and that of low mol wt LAP completely destroyed by treatment with butanol, presumably owing to denaturation. No return of activity was observed following dialysis to remove butanol.

The results suggest that the increase in activity of liver ALP in serum following treatment with butanol was not a consequence of conversion of high mol wt ALP into the liver isoenzyme by the solvent. It seems on the contrary to have stemmed from an independent enhancing effect of butanol on the liver isoenzyme itself. High

Figure 4.12. ALP isoenzyme patterns in 2.5% polyacrylamide gel showing the effects of butanol extraction 1) purified liver isoenzyme 2) liver isoenzyme + butanol 3) liver isoenzyme + butanol after dialysis 4) purified high mol wt ALP 5) high mol wt ALP + butanol 6) high mol wt ALP + butanol after dialysis.

TABLE 4.9.

Quantitative effect of butanol extraction on partially purified low and high mol wt ALP, γGT and LAP

Isoenzyme	Enzyme activities (iu/l)					
	ALP		γGT		LAP	
	Control	Butanol-treated	Control	Butanol-treated	Control	Butanol-treated
Low mol wt	73	78	7.4	3.1	3.7	0
High mol wt	40	3	9.7	0	2.1	0

mol wt ALP may have been extracted into the organic layer and there denatured. Similar conclusions apply to γGT, and LAP, although these also suffered partial or complete denaturation of their low mol wt isoenzymes by the harsh treatment of this organic solvent.

The experiment was repeated using, instead of butanol alone, a mixture of butanol and di-isopropyl ether (40:60 v/v) which has been recommended as having less of a denaturing effect on proteins and enzymes (Cham and Knowles, 1976). In this experiment the high mol wt component from bile was also included. However, although the use of this gentler solvent mixture did preserve low mol wt γGT and LAP from denaturation, the effect on the high mol wt components of all three enzymes from both serum and bile was the same i.e. they disappeared entirely

with no reappearance elsewhere in the gel. Either they were denatured in the aqueous layer (unlikely since under these conditions none of the low mol wt components of the three enzymes were denatured) or, more likely, they were extracted into the organic layer, being lipid soluble, and there denatured by the high concentration of solvent.

Various conflicting findings have been reported for the effect of butanol on ALP in serum. Price et al (1972) found an overall activation whereas Walker and Pollard (1971) found that many sera showed a loss in activity although some showed striking increases. As far as high mol wt ALP itself is concerned, most reports agree that its activity disappears (Jennings et al, 1970; Walker and Pollard, 1971; Price et al, 1972; Epstein et al, 1978). Opinions vary as to whether or not it is converted to a lower mol wt form. Jennings et al (1970), using Sephadex G200 chromatography, concluded that it was, although activity was frequently very low owing to denaturation. Walker and Pollard (1971) sometimes observed conversion of the origin activity on 5% polyacrylamide gel electrophoresis into faster diffuse bands running behind (not with) the liver isoenzyme, but more often no trace of activity appeared elsewhere in the gel. Fritsche and Adams-Park (1974) found that high mol wt ALP, running in

the α_1 globulin position on cellulose acetate electrophoresis, remained at the origin after butanol extraction, possibly indicating some degree of denaturation. Price et al (1972) observed conversion of high mol wt biliary ALP into a lower mol wt form. Only one study has been done on enzymes other than ALP, namely high mol wt γGT in bile (Wenham et al, 1978). Here butanol caused considerable loss of activity but appeared to result in some conversion into a lower mol wt form.

In summary, the conflicting evidence suggests that this powerful organic solvent has effects of varying severity on enzyme structure under different extraction conditions. Possibly the source and purity of the butanol may also be a contributory factor. In the present study, high mol wt ALP, γGT and LAP from serum and bile were all apparently denatured by butanol without conversion to a lower mol wt form. It is probable that they were extracted into the butanol layer together with the lipid. This suggests that they consist of enzymes attached to lipid, lipoprotein or membrane fragments. Since all three enzymes are membrane-bound in the liver, the latter possibility seems the most likely.

4.7.2. Treatment with Triton X-100

Triton X-100 (polyoxyethylene glycol (9-10) p-t-

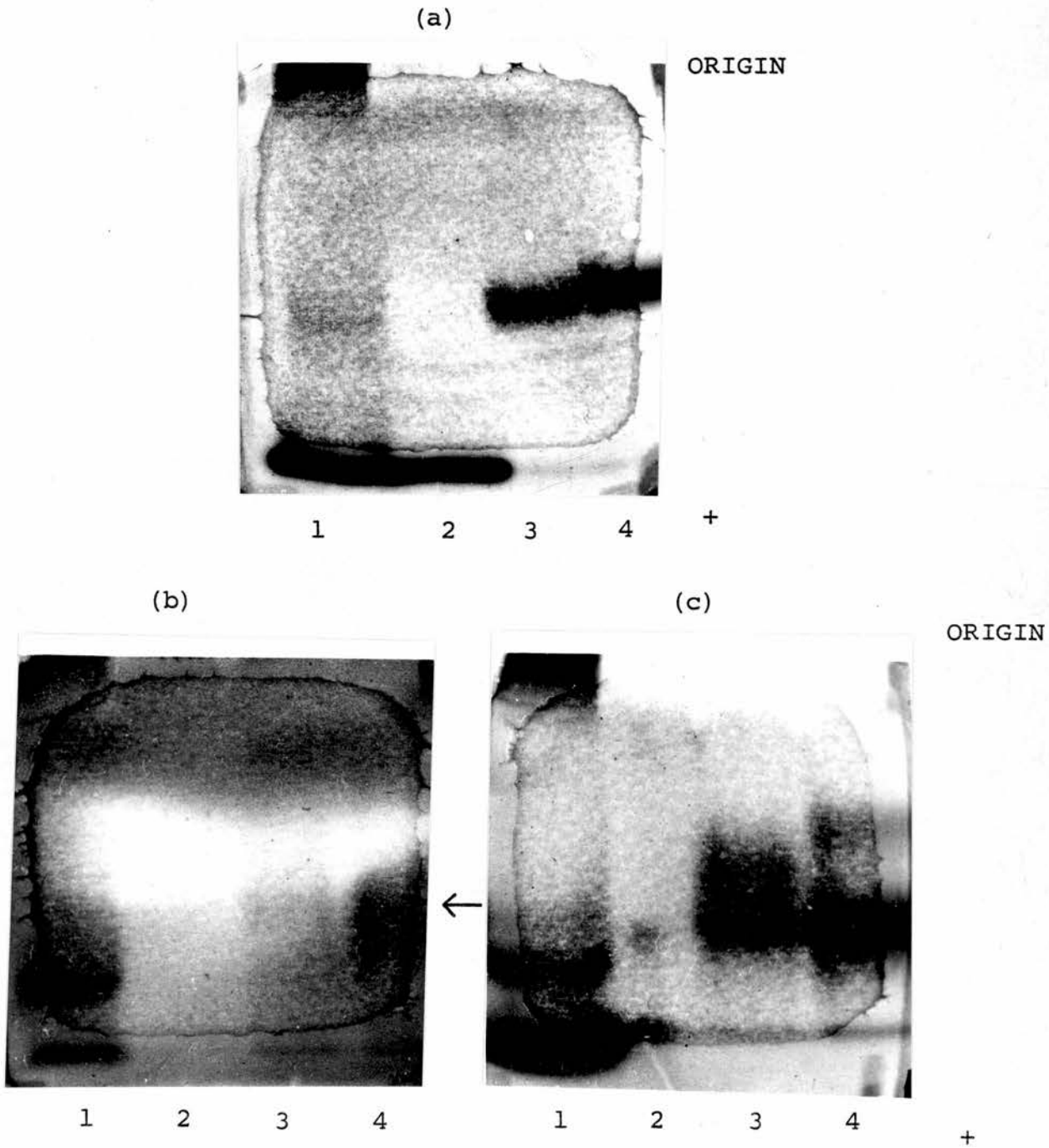
octyl-phenol) is a relatively mild non-ionic detergent which has frequently been used to solubilise membrane proteins (Sohn and Marinetti, 1974; Helenius and Simons, 1975). Since the working hypothesis being tested was that high mol wt ALP, γGT and LAP represented membrane fragments, the solubilising effects of Triton X-100 on these complexes were assessed.

The following samples were incubated with 0.2% Triton X-100 in a 1:1 (v:v) ratio for 24 hours at 4°C:

1) serum containing liver and high mol wt ALP
2) partially purified liver ALP
3) partially purified high mol wt serum ALP
4) partially purified high mol wt biliary ALP.

Comparison of detergent-treated with control samples following electrophoresis in 2.5% polyacrylamide gel showed that Triton X-100 had altered the mobility of all three high mol wt enzymes (i.e. ALP, γGT, LAP) from serum and bile to a similar extent. They had penetrated further into the gel, to a position intermediate between that of the untreated high mol wt enzymes and that of the low mol wt or liver isoenzymes (Fig 4.13.). The purified high mol wt enzymes formed a single band after detergent treatment but the unpurified high mol wt enzymes in whole serum appeared to be partially protected from the effects of the

Figure 4.13. Detergent-altered isoenzyme patterns in 2.5% polyacrylamide gel showing the effects of Triton X-100. a) ALP b) γGT c) LAP. 1) serum containing low and high mol wt forms 2) purified liver ALP 3) purified high mol wt form from serum 4) purified high mol wt form from bile. [N.B. in gel (b) the staining in the upper part of the gel is orange, non-specific background staining. The position of the pink bands is marked with an arrow.]



detergent so that some of the original high mol wt enzymes remained. In the case of γGT and LAP one extra band was formed in the detergent-treated serum, running just behind the normal low mol wt isoenzyme band.

The mol wt of the new bands, estimated by 4 to 26% polyacrylamide gradient gel electrophoresis, was intermediate between that of the original high mol wt enzymes and that of the normal liver or low mol wt isoenzymes. An exact value could not be determined because Triton X-100, being a non-ionic detergent, could not be introduced into the gradient gel even by prolonged electrophoresis for several days. Some degree of reassociation may therefore have taken place in the detergent-treated samples as they passed through the gel, leading to a smear of activity through a range of gel concentrations (Figs 4.14. and 4.15.). The average mol wt was approximately 660 000 and was certainly greater than that of liver ALP (240 000). This is in agreement with recent observations of Epstein et al (1978), using whole serum, that high mol wt ALP was converted by Triton X-100 to lower mol wt forms whose size was still greater than that of the liver isoenzyme.

In conclusion, Triton X-100 had no effect on the low mol wt isoenzymes of ALP, γGT and LAP but it converted the purified high mol wt components of these three

Figure 4.14. Effect of the detergents Triton X-100 and SDS on ALP isoenzymes in purified preparations of 1) liver ALP 2) high mol wt ALP from serum 3) high mol wt ALP from bile. Electrophoresis was to equilibrium in 4 to 26% polyacrylamide gradient gels. The serum proteins are shown for comparison.

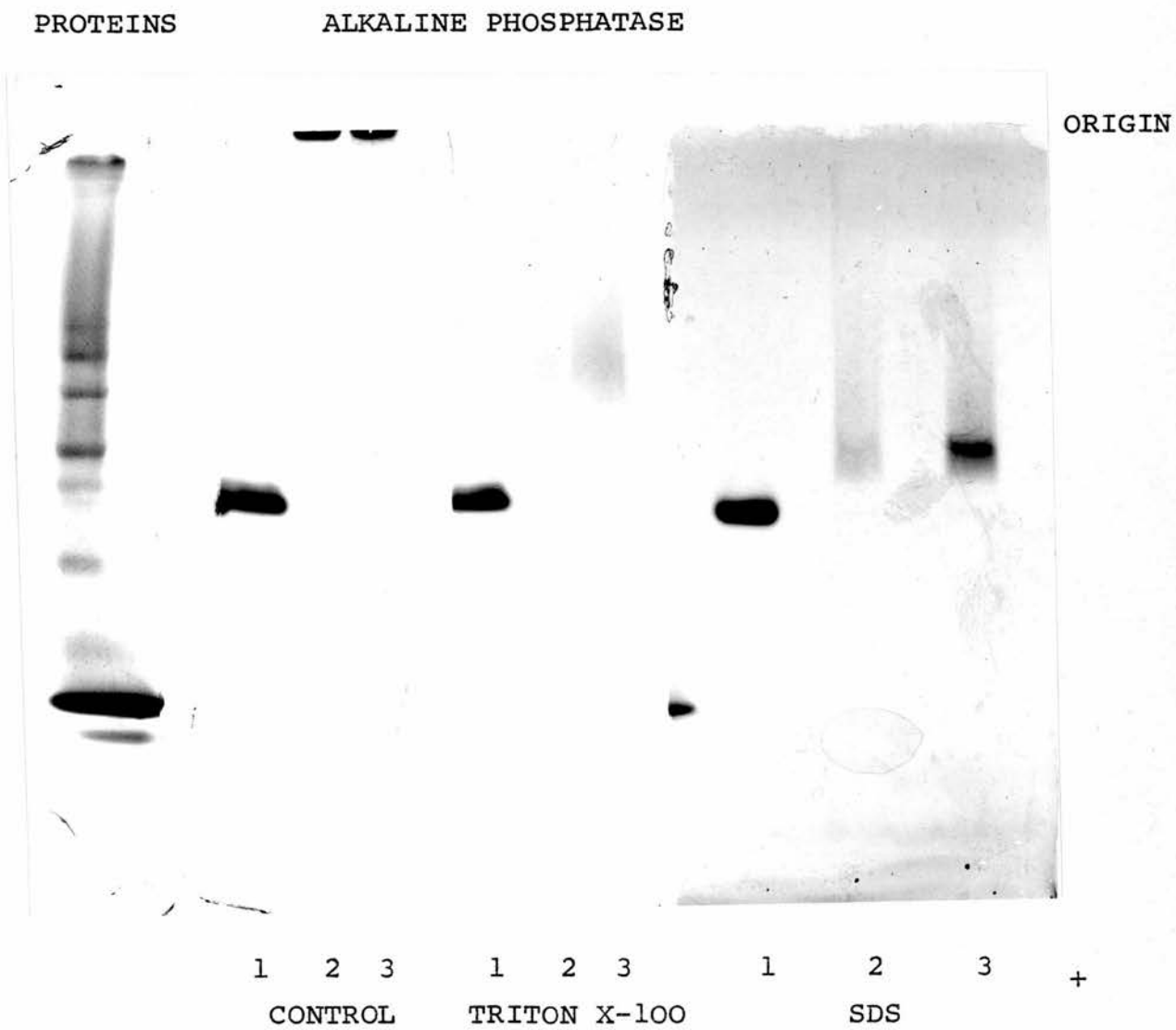
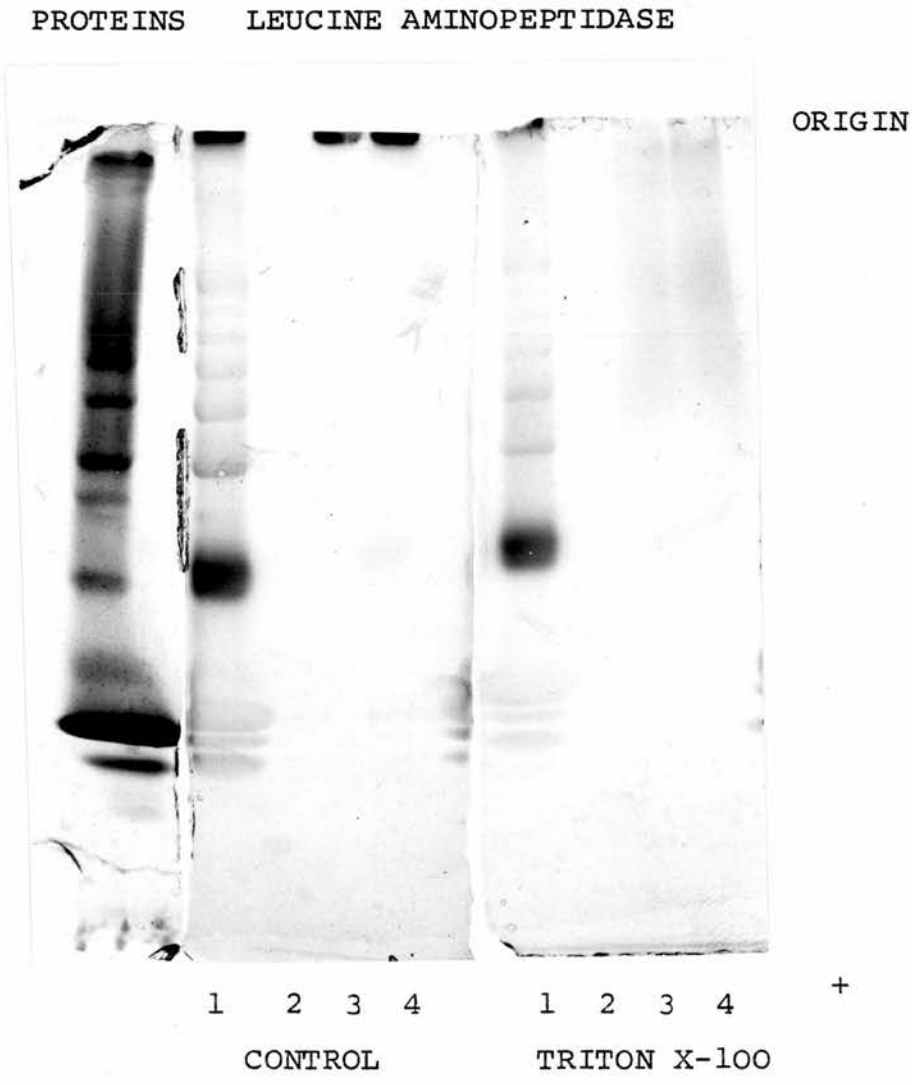


Figure 4.15. Effect of Triton X-100 on LAP isoenzymes in 1) serum from a patient with liver disease 2) purified liver ALP (no LAP present) 3) purified high mol wt component from serum and 4) purified high mol wt component from bile. Electrophoresis was to equilibrium in 4 to 26% polyacrylamide gradient gels. The serum proteins are shown for comparison.[The faint bands observed in the gels stained for LAP are coloured blue, not pink, and correspond to protein stained by diffusion of the amido black from the neighbouring gel].



enzymes from both serum and bile to lower mol wt forms of apparently identical mol wt (approximately 660 000). The nature of these lower mol wt forms is discussed in Chapter 7.

4.7.3. Treatment with sodium dodecyl sulphate (SDS) and mercaptoethanol

SDS is an anionic detergent which, like Triton X-100, has often been used to solubilise membrane proteins. The more hydrophilic nature of its polar groups renders it a more powerful solubilising agent than Triton X-100 but because it frequently disrupts the quaternary structure of the membrane protein, it is more likely to cause denaturation (Sohn and Marinetti, 1974; Helenius and Simons, 1975). Mercaptoethanol is generally included with SDS as a reducing agent to prevent disulphide bridges being formed between polypeptide chains and consequent reassociation.

The same samples as in the previous section were incubated with a solution of 2% SDS:0.2% mercaptoethanol in the ratio of 1:1 (v:v) at 37°C for 5 minutes, at room temperature for 30 minutes and overnight at 4°C. This protocol was used to maximise solubilisation and minimise denaturation. Nevertheless SDS inactivated both γGT and LAP completely, presumably by interfering with their quaternary structure. Therefore no results are available

for these enzymes.

Electrophoresis of detergent-treated and control sera in 2.5% polyacrylamide gel showed that liver ALP was unaffected by SDS but the high mol wt ALPs from serum and bile were both converted to lower mol wt forms which migrated with the mobility of the liver isoenzyme (Fig 4.16.). The mol wts of these new forms were estimated by electrophoresis to equilibrium in 4 to 26% polyacrylamide gradient gel pre-equilibrated with 0.1% SDS, and found to be 410 000 for the solubilised complexes from both serum and bile c.f. liver and low mol wt biliary ALP with mol wts of 240 000 (Figs 4.14. and 4.17.). By contrast, Akedo et al (1967), using chloroform-methanol and SDS, converted high mol wt into a form of only slightly lower mol wt, which was still excluded from a Sephadex G200 gel.

In conclusion, like Triton X-100, SDS had no effect on liver ALP but converted high mol wt ALP from both serum and bile to smaller forms of identical mol wt (410 000). This was still higher than the mol wt of the liver and low mol wt biliary isoenzymes. The nature of this detergent-solubilised enzyme is discussed in Chapter 7. Since both Triton X-100 and SDS, which interfere with hydrophobic bonding, disrupted the structure of the high mol wt enzymes. it seems likely that hydrophobic bonding plays a

Figure 4.16. Detergent-altered ALP isoenzyme patterns in 2.5% polyacrylamide gel showing the effects of SDS. 1) serum containing low and high mol wt ALP 2) purified liver ALP 3) purified high mol wt ALP from serum 4) purified high mol wt ALP from bile.

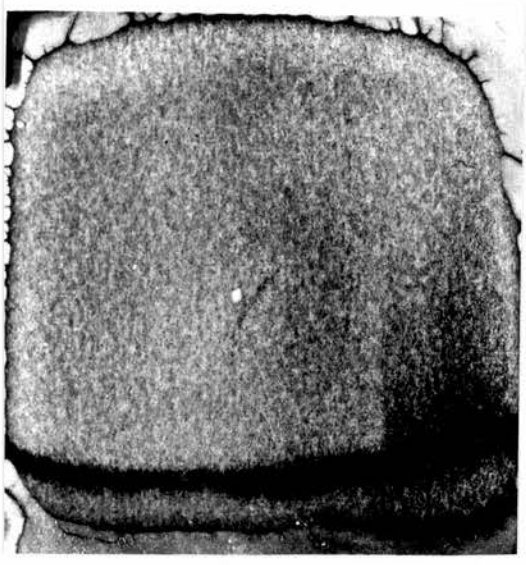
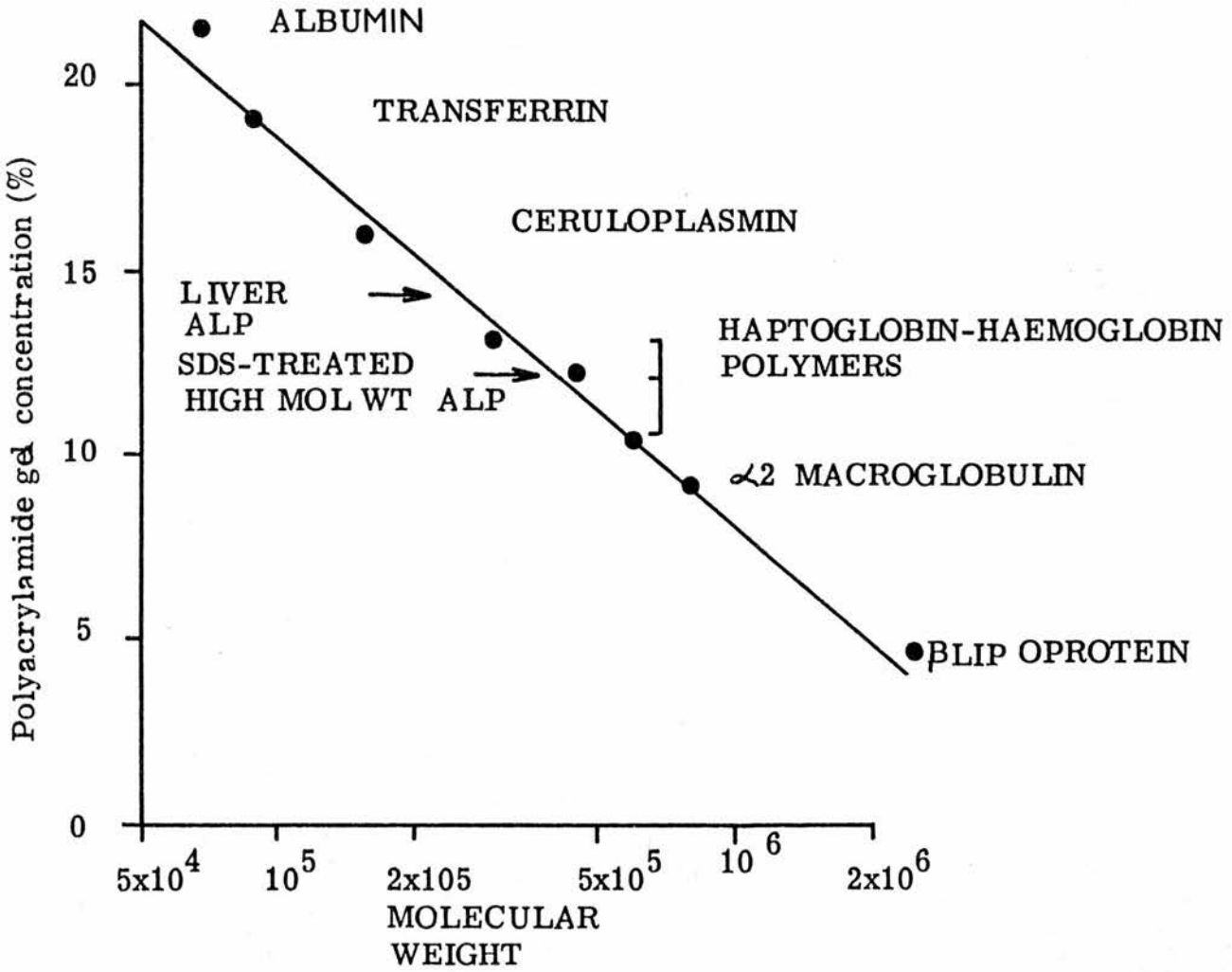


Figure 4.17. Semi-logarithmic calibration plot of polyacrylamide gel concentration versus mol wt in a 4 to 26% polyacrylamide gradient gel. The positions of liver ALP and SDS-treated high mol wt ALP are shown.



large part in maintaining that structure. This is consistent with the theory that they represent membrane fragments.

4.8. RELATIONSHIP OF THE HIGH MOLECULAR WEIGHT ENZYMES WITH LIPOPROTEIN X

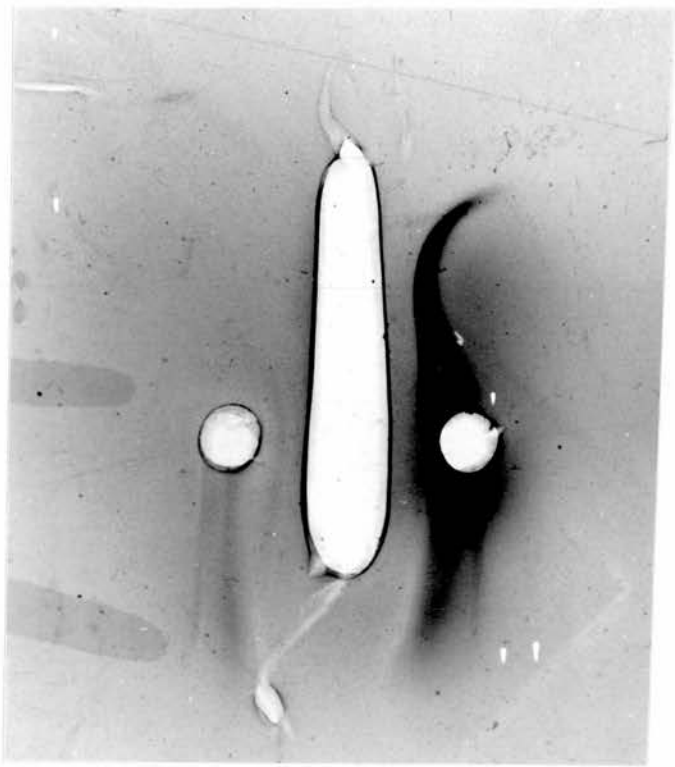
Like high mol wt ALP, LPX is released into the circulation in cholestatic liver disease. Recent experiments using electron microscopy have revealed that morphologically LPX resembles stacked membranous vesicles (Hauser et al, 1977). It was thought possible that LPX and high mol wt ALP, γGT and LAP might be related or even identical. Experiments were therefore directed towards establishing whether or not a relationship existed.

4.8.1. Qualitative evidence for association of lipoprotein X with membrane marker enzymes

As a pilot experiment, LPX immunoelectrophoresis was performed in duplicate on a) serum from a patient with obstructive jaundice and b) a control serum from a patient with no evidence of liver disease. After washing in saline for 2 days, any precipitin arcs formed were stained for protein and ALP respectively. Only serum (a) gave a positive LPX precipitin arc and this stained strongly for ALP (Fig 4.18.).

More detailed investigations were then carried out

Figure 4.18. LPX precipitin arc stained for ALP in a serum from a patient with obstructive jaundice (a). No precipitin arc in the control serum (b).



on four sera from patients with obstructive jaundice and a control serum. These were subjected to immunoelectrophoresis in quadruplicate by the method of Scheidegger (1955) against the antisera listed in Table 4.10. After washing in saline for 2 days, any precipitin arcs formed were stained for a) protein b) ALP c) γGT and d) LAP.

Only the LPX (Fig 4.19.) and βlipoprotein (Fig 4.20) precipitin arcs stained for enzyme activity; furthermore this staining was present only in the sera from the patients with obstructive jaundice and not in the control serum (Table 4.10.). For γGT and LAP, where Fast Garnet GBC was used as a stain, the colour of the LPX precipitin arcs was pink whereas the colour of the anodal 'tails' (Fig 4.19.) and that of the βlipoprotein arcs (Fig 4.20.) was orange, the colour of the unreacted dye. In order to check whether any of the precipitin arcs stained as a result of non-specific dye uptake, the experiment was repeated omitting the enzyme substrates. No non-specific dye uptake was observed.

The antiserum against LPX is known to cross-react with other proteins and lipoproteins in serum. LPX is therefore identified by its characteristic migration towards the cathode under the conditions of the assay, whereas the other proteins and lipoproteins, including

TABLE 4.10.

Presence or absence of enzyme staining associated with precipitin arcs following immunoelectrophoresis of a control serum and 4 obstructive jaundice sera against various antisera

Antiserum	Obstructive jaundice sera				Control serum			
	Protein	ALP	ɣGT	LAP	Protein	ALP	ɣGT	LAP
IgG	+	-	-	-	+	-	-	-
IgA	+	-	-	-	+	-	-	-
IgM	+	-	-	-	+	-	-	-
Whole human serum	+	-	-	-	+	-	-	-
βlipoprotein	+	+	+	+	+	-	-	-
LPX	+	+	+	+	-	-	-	-

Figure 4.19. LPX immunoelectrophoresis plate stained for a) ALP b) γGT and c) LAP. The arcs on the cathodal side of the origin correspond to LPX. Samples 1 and 6 are control sera; samples 2 to 5 are sera from patients with obstructive jaundice. [N.B. The staining on the anodal side of the origin in gels (b) and (c) is orange compared to the pink colour of the LPX precipitin arcs].

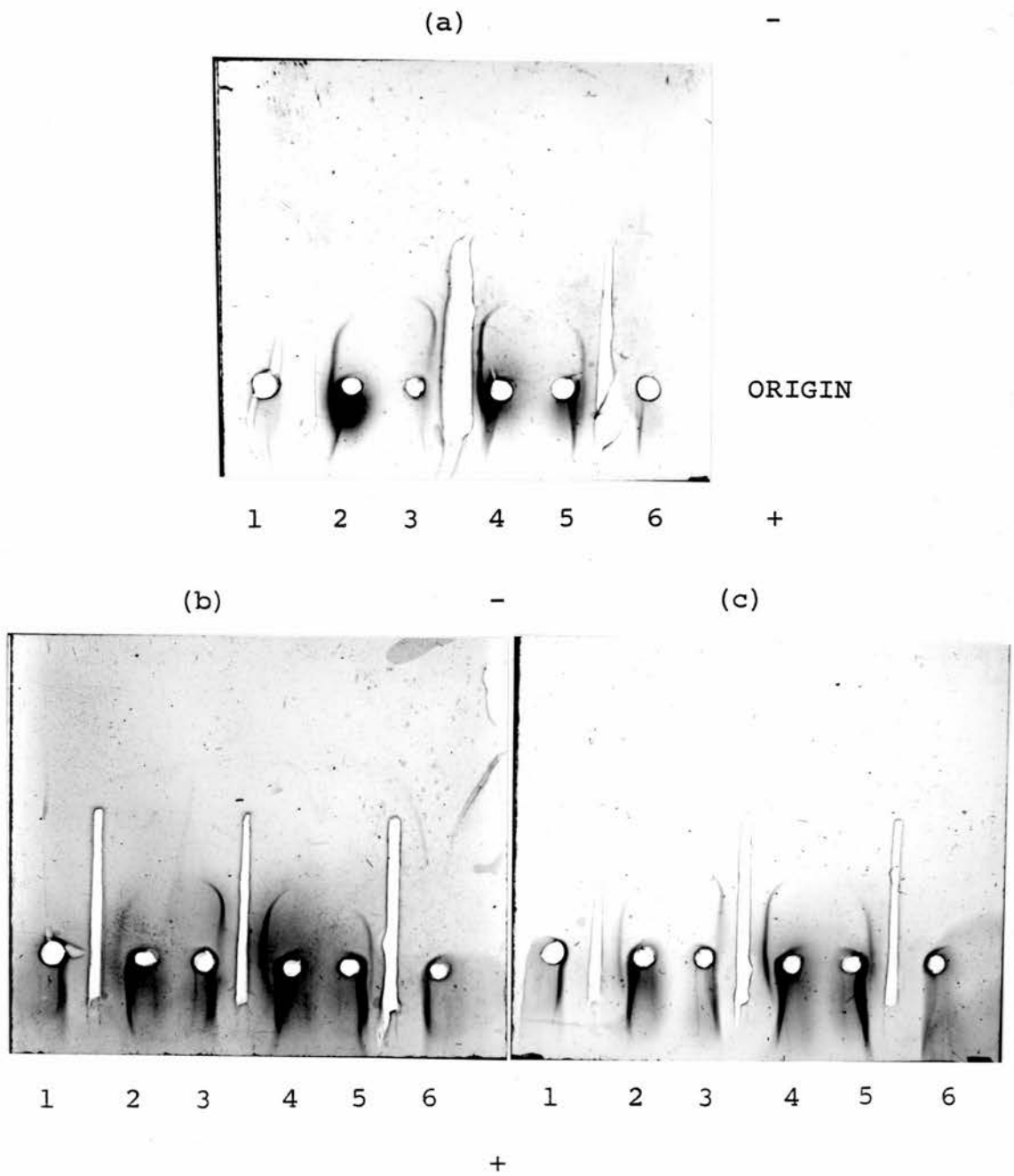
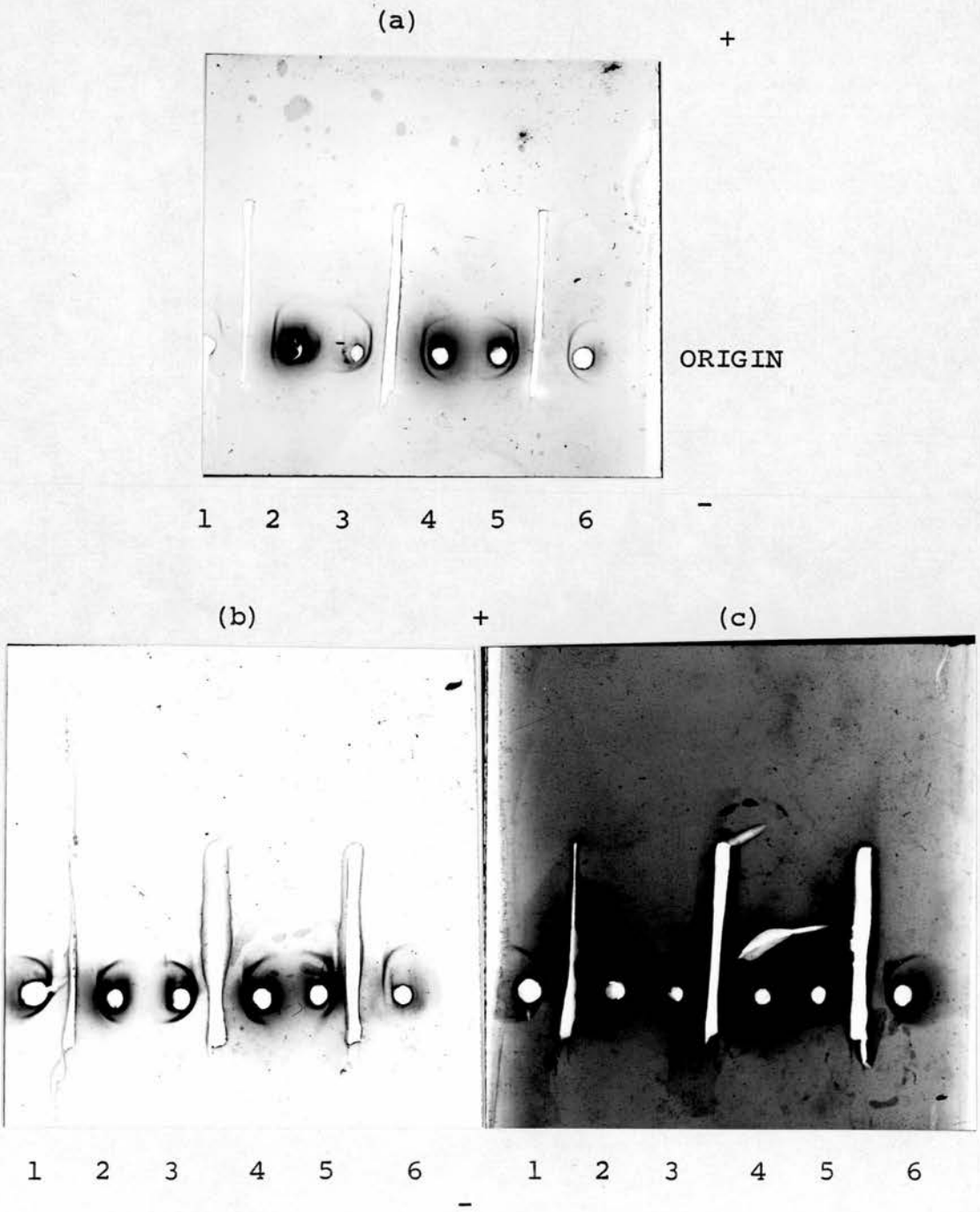


Figure 4.20. βLP immunoelectrophoresis plate stained for a) ALP b) γGT and c) LAP. Samples 1 and 6 are control sera; samples 2 to 5 are sera from patients with obstructive jaundice. [N.B. the staining in gels (b) and (c) is orange].



βlipoprotein, migrate towards the anode. However, to add to the problem, LPX may migrate to the anode under certain conditions e.g. after prolonged storage or in the presence of high concentrations of bile salts (Manzato et al, 1976). There are therefore at least three possible explanations for the observations:

1) The enzymes are associated with LPX and not with βlipoprotein but some LPX migrates anodally and cross-reacts with βlipoprotein antiserum.

2) Some association may exist between the enzymes and βlipoprotein as well as between the enzymes and LPX. The observations of Lawrence and Melnick (1961) support this theory. They found that the βlipoprotein precipitin line stained not only for ALP and LAP (as shown here) but also for many other enzymes originating both from membrane and from cytoplasm. Isolation of βlipoprotein by ultra-centrifugation, followed by sonication resulted in activation of these enzymes. The authors proposed that many enzymes were non-specifically sequestered in serum βlipoprotein. This explanation is in keeping with the ALP band which migrates in the βlipoprotein position during starch gel electrophoresis (Chiandussi et al, 1962; Hodson et al, 1962). As explained in section 2.10, although such a βlipoprotein-associated ALP would appear in the void

volume during Sephadex G200 chromatography, it would not be excluded by the larger mesh gels used in the Sepharose range. It is therefore not included in the definition of the high mol wt ALP which is specific for liver disease and which forms the subject of this thesis.

3) The different colour of the staining of the anodal precipitates may suggest that this staining was an artefact and did not reflect enzyme activity. In keeping with this theory, Seidel et al (1970) took the view that the anodal 'tails' represented non-specific precipitation such as occurs with any serum in agar gel. A different approach was required to resolve the question.

4.8.2. Qualitative evidence for association of lipoprotein X with high molecular weight components of membrane marker enzymes

From the sera of 72 patients with liver disease (Chapter 5), 7 sera were selected which had strongly positive LPX precipitin arcs. All of these 7 sera had high mol wt ALP activities greater than 36 iu/l (approximate upper limit of reference range: 10 iu/l). This was circumstantial evidence that the two might be related.

When partially purified preparations of liver and high mol wt ALP from serum and bile were subjected to immunoelectrophoresis against LPX and βlipoprotein

antisera, no precipitin arcs were found although general precipitation of protein staining for ALP activity around the sample well was observed. No such precipitation occurred for the liver isoenzyme. The absence of a cathodal precipitin arc may have been because the antisera used had a low titre and were insensitive to low concentrations of antigen. Alternatively, the physical characteristics of LPX or βlipoprotein may have been altered during the purification process so that they did not migrate during electrophoresis. The problem therefore had to be approached indirectly.

The following samples were incubated for 24 hours at 4°C with a) LPX antiserum b) βlipoprotein antiserum and c) saline (as controls) in the ratio of 1 volume sample: 2 volumes antiserum:

1) serum containing liver and high mol wt ALP
2) partially purified liver ALP
3) partially purified high mol wt serum ALP
4) partially purified high mol wt biliary ALP.

Electrophoresis of the supernatants in 2.5% polyacrylamide gel (Fig 4.21.) and 4 to 26% polyacrylamide gradient gel (Fig 4.22.), followed by staining for ALP, γGT and LAP, showed that incubation with LPX and βlipoprotein antisera had no effect on the low mol wt isoenzymes of ALP,

Figure 4.21.(a). ALP isoenzyme patterns in 2.5% polyacrylamide gel showing the effects of precipitation by antisera. 1) purified liver ALP + saline, 2) purified high mol wt ALP from serum + saline 3) LPX antiserum alone 4) liver ALP + LPX antiserum 5) high mol wt ALP + LPX antiserum 6) LPX antiserum alone 7) βLP antiserum alone 8) liver ALP + βLP antiserum 9) high mol wt ALP + βLP antiserum 10) βLP antiserum alone.

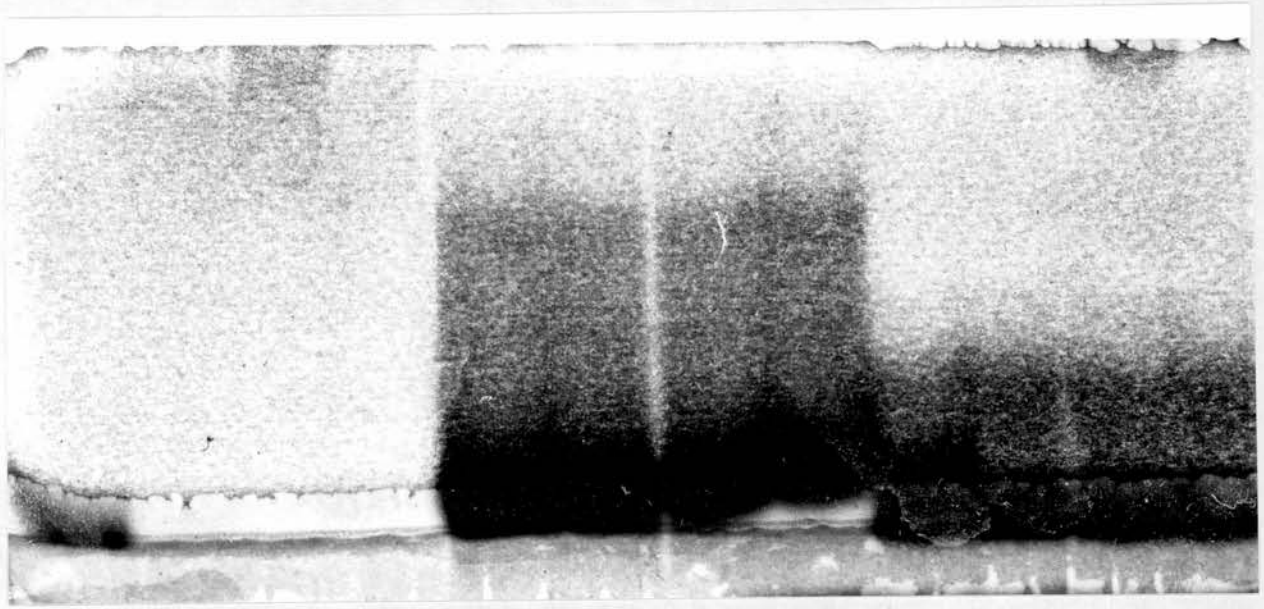


Figure 4.21.(b). LAP isoenzyme patterns in 2.5% polyacrylamide gel showing the effects of precipitation by antisera. 1) LPX antiserum alone 2) βLP antiserum alone 3) purified high mol wt component from serum + saline 4) purified high mol wt component from serum + LPX antiserum 5) purified high mol wt component from serum + βLP antiserum 6) purified high mol wt component from bile + saline 7) purified high mol wt component from bile + LPX antiserum 8) purified high mol wt component from bile + βLP antiserum.

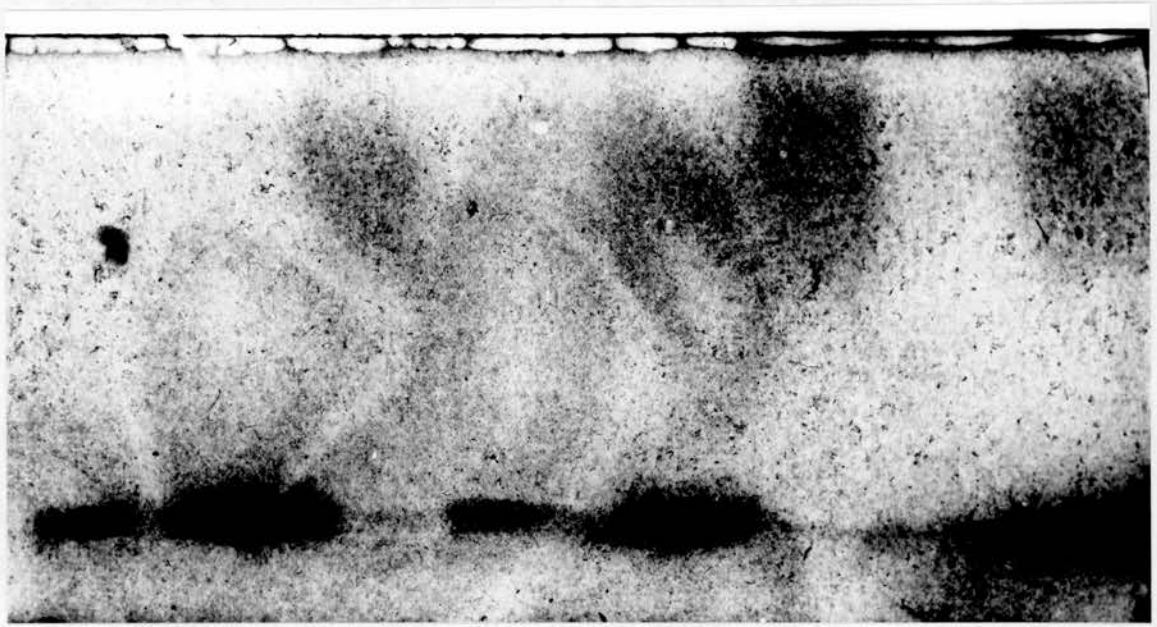
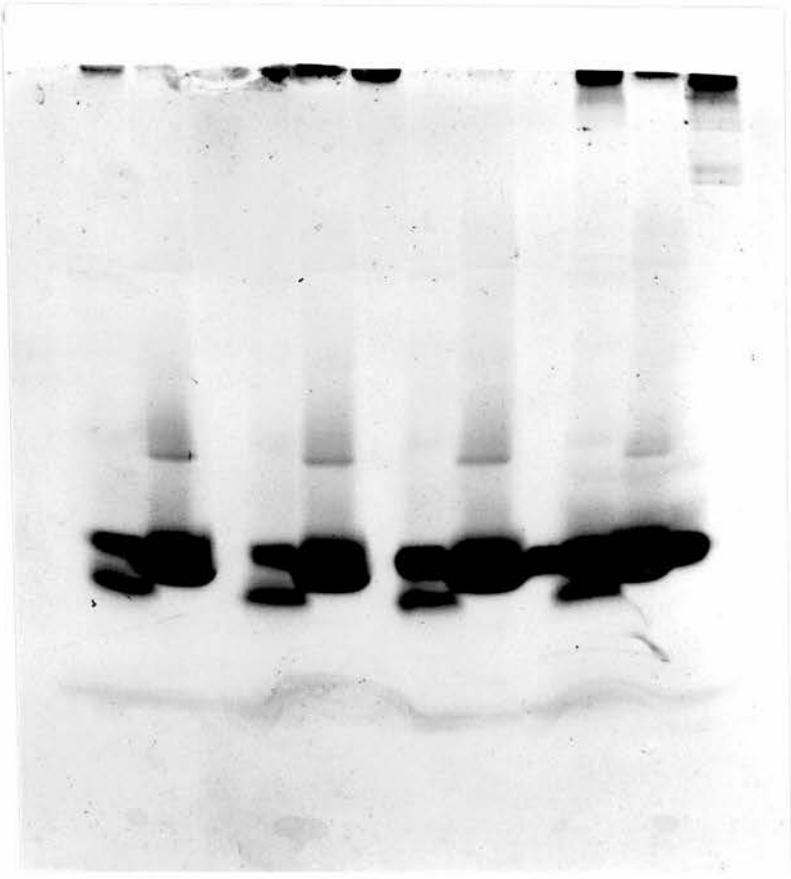


Figure 4.22. ALP isoenzyme patterns in 4 to 26% polyacrylamide gradient gel showing the effects of precipitation by antisera. 1) whole serum containing liver and high mol wt ALP + saline 2) whole serum + LPX antiserum 3) whole serum + βLP antiserum 4) purified liver ALP + saline 5) liver ALP + LPX antiserum 6) liver ALP + βLP antiserum 7) purified high mol wt component from serum + saline 8) high mol wt component (serum) + LPX antiserum 9) high mol wt component (serum) + βLP antiserum 10) purified high mol wt component from bile + saline 11) high mol wt component (bile) + LPX antiserum 12) high mol wt component (bile) + βLP antiserum.
Similar patterns were observed for γGT and LAP.
[N.B. The ALP precipitates formed in incubations (2), (8) and (11) were more obviously excluded at the origin in the original gel than in this photograph, in which the top of the gel has become slightly displaced].



γGT and LAP. However, incubation of the high mol wt components from both serum and bile with LPX antiserum resulted in the disappearance of the high mol wt components of all three enzymes. Immunoelectrophoresis showed that this was accompanied by the disappearance of the LPX precipitin arc which had stained for all three enzymes. It was also accompanied by the disappearance of the non-specific anodal precipitate (LPX immunoelectrophoresis) and of the βlipoprotein precipitin arc (βlipoprotein immuno-electrophoresis). By contrast, incubation of the high mol wt components with βlipoprotein antiserum had no effect on the activity of the high mol wt enzymes. Immunoelectro-phoresis showed that both the non-specific anodal precipitate (LPX immunoelectrophoresis) and the βlipoprotein precipitin arc (βlipoprotein immunoelectrophoresis) had largely disappeared. The LPX precipitin arc was unaffected. These observations are summarised in Tables 4.11. and 4.12.

The immunoelectrophoretic evidence suggests that the LPX antiserum employed did indeed cross-react with βlipoprotein but that the βlipoprotein antiserum employed gave no cross-reaction with LPX. The balance of the evidence strongly suggests that the high mol wt components of all three enzymes from serum and bile are associated with LPX in the form of a complex and not with βlipoprotein

TABLE 4.11.

Effect of incubation with a) saline b) βlipoprotein antiserum and c) LPX antiserum on low and high mol wt ALP, ɣGT and LAP

Enzyme component	Saline	Incubated with antisera to: βlipoprotein	LPX
Low mol wt (serum)	Unchanged	Unchanged	Unchanged
High mol wt (serum)	Unchanged	Unchanged	Precipitated
High mol wt (bile)	Unchanged	Unchanged	Precipitated

TABLE 4.12.

Effect of incubation with a) saline b) βlipoprotein antiserum and c) LPX antiserum on the protein and enzyme staining associated with the LPX precipitin arc, non-specific anodal precipitation and the βlipoprotein arc, following immunoelectrophoresis

Precipitate	Presence(+) or absence(-) of precipitate: Saline incubations	βLP antiserum incubations	LPX antiserum incubations
LPX arc (cathodal)	+	+	-
LPX precipitate (anodal)	+	-	-
βLP arc (anodal)	+	-	-

since they are precipitated by LPX antiserum but not by βlipoprotein antiserum. Therefore, although there may be some non-specific association between the enzymes and βlipoprotein, the high mol wt enzymes, as defined in this thesis, are not involved in the association.

4.8.3. Semi-quantitative evidence: association of membrane marker enzymes with lipoprotein X

From the sera of 72 patients with liver disease (Chapter 5), 13 sera with positive LPX were selected. Following immunoelectrophoresis, the LPX precipitin arcs were stained for protein, ALP, γGT and LAP. The depth of staining was rated on a scale from 1 to 3 (Table 4.13.).

TABLE 4.13.

Depth of protein and enzyme staining (scored on a scale from 1 to 3) associated with LPX precipitin arcs in 13 sera

Serum	Protein	ALP	γGT	LAP
1	2	1	2	3
2	2	1	1	1
3	2	1	2	1
4	3	2	3	3
5	2	1	1	1
6	3	2	3	3
7	3	2	1	2
8	2	1	2	2
9	3	2	2	1
10	3	2	2	3
11	3	2	2	3
12	3	1	2	2
13	3	2	2	2

Correlation procedures were unsuitable because of the large number of ties in a small sample. Instead a t-test between each protein/enzyme pair was carried out. As an example, protein staining was divided into two categories (score 2 and score 3). The t-test was then performed on the ALP scores of each of these two categories to see whether or not the ALP scores were significantly higher in the score 3 protein category than in the score 2 protein category.

TABLE 4.14.

One-tailed associated probabilities in t-test to compare depths of protein and enzyme staining associated with LPX precipitin arcs in 13 sera. See text for details

Scores divided into 2 groups	Scores associated with each group			
	Protein	ALP	LAP	ɣGT
Protein		.0001	.06	.08
ALP			.06	.10
LAP				.015
ɣGT				

Table 4.14 shows the one-tailed probabilities associated with the calculated t values. In each case, a positive relationship between depth of staining of LPX-associated protein and enzymes was found but this only reached significance ($p < 0.05$) for the protein-ALP and

γGT-LAP pairs. However, the associated probabilities were sufficiently low to suggest that if the number of sera studied had been increased to, say, 20 the t values for all the pairs might have achieved significance in this relatively crude test of association. It therefore seems likely that sera with a high LPX (estimated by protein content) are also likely to have large amounts of enzyme associated with the LPX.

Approaching the problem rather differently, high mol wt ALP was measured in the 13 sera, and also in an additional 4 sera which scored 1 for protein staining of the LPX precipitin arcs. The protein scores were divided into three categories and one-way analysis of variance was carried out on the high mol wt ALP activities (logarithmically transformed to normalise the data) in each category (Table 4.15.). The results show that patients with large amounts of LPX are also likely to have high activities of high mol wt ALP ($p < 0.005$).

If a t-test is carried out between LPX-associated ALP and high mol wt ALP (Fig 4.23.), the geometric means of the classes scoring 1 and 2 for LPX-associated ALP are 21 and 71 iu/l high mol wt ALP respectively ($p < 0.05$). Therefore patients with large amounts of LPX-associated ALP are also likely to have high activities of high mol wt ALP.

Figure 4.23. High mol wt ALP activities in sera showing weak (± or +) and strong (++) staining of LPX-associated ALP.

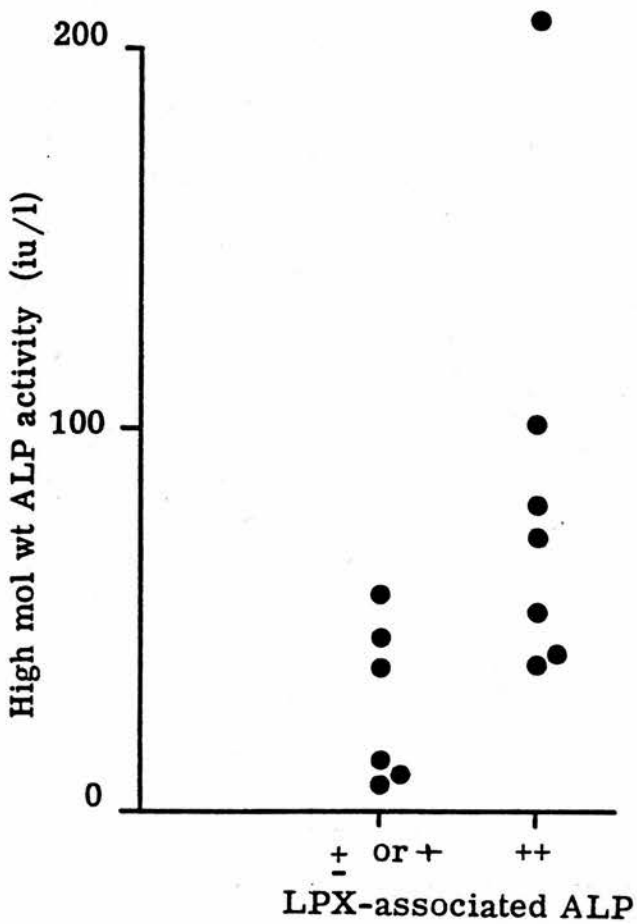


TABLE 4.15.

High mol wt ALP activities measured in 17 sera containing LPX

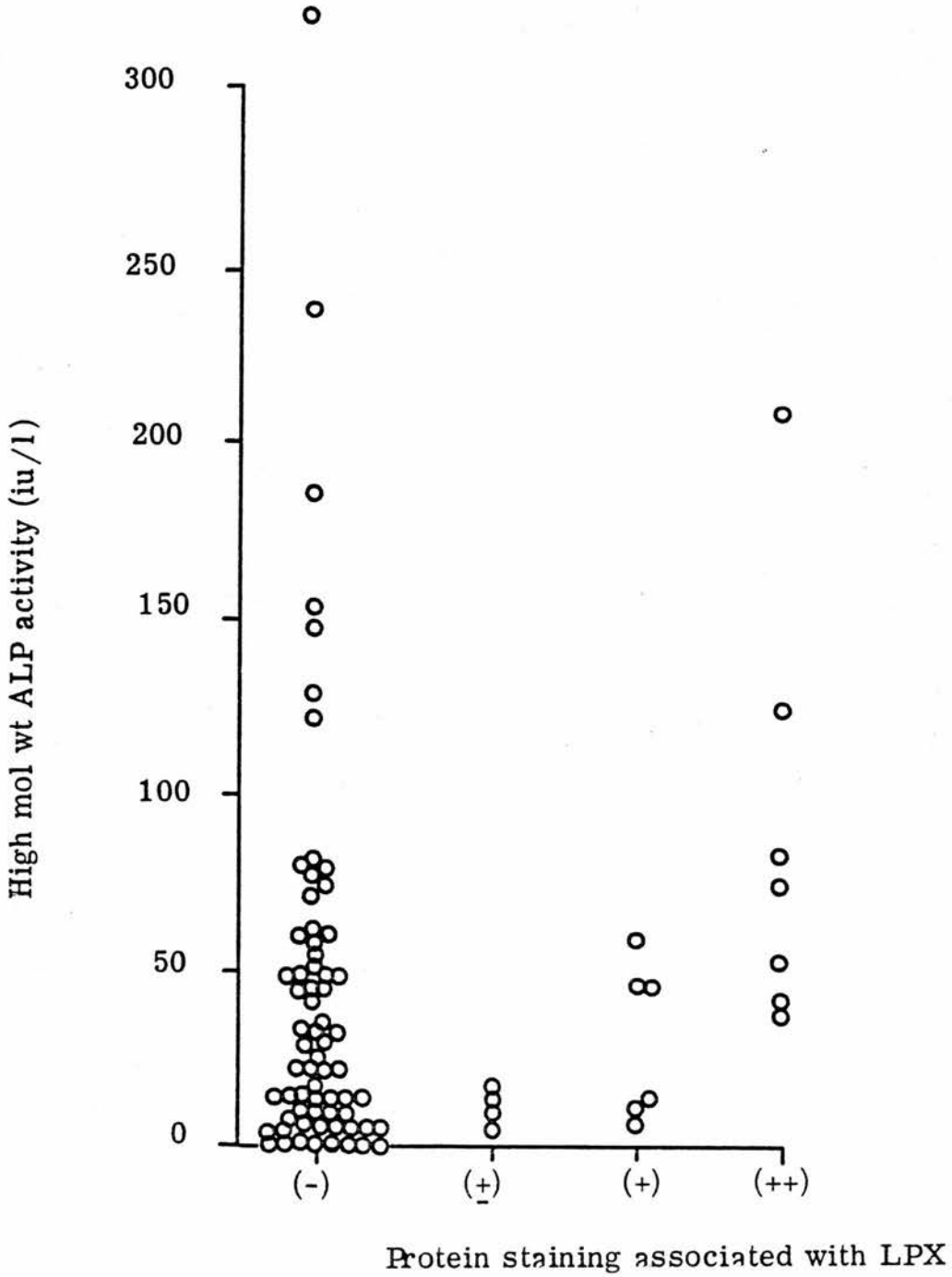
	Depth of LPX protein stain		
	1	2	3
N	4	6	7
Geometric mean	10 iu/l	22 iu/l	73 iu/l
Range	6-16 iu/l	7-57 iu/l	37-207 iu/l
Two-tailed probability associated with F value		<0.005	
Two-tailed probabilities for differences between means	<0.05	<0.005	<0.001

* The t-test was carried out for each pair of LPX categories as shown.

However, the converse of these statements is not true. Fig 4.24. shows that high mol wt ALP frequently rose to high levels of activity in the absence of detectable LPX (and therefore of detectable LPX-associated enzyme). Brocklehurst et al (1978) have also found that the origin band on starch gel electrophoresis may occur in the absence of detectable LPX.

In summary, patients with large amounts of LPX were likely to have large amounts of enzyme associated with LPX

Figure 4.24. High mol wt ALP activities in sera containing various amounts of LPX as shown by depth of precipitin arc staining for protein.



and large amounts of high mol wt ALP. On the other hand, patients with large amounts of high mol wt ALP did not necessarily have large amounts of LPX. The experiments were not sufficiently quantitative to establish conclusively whether or not LPX has a constant enzyme composition.

4.9. SUMMARY OF INVESTIGATIONS INTO THE NATURE OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE

1. High mol wt ALPs from serum and bile both have probably identical mol wts of greater than 1 million and carry an identical net positive charge which is greater than any other isoenzyme tested at alkaline pH. Since the electrophoretic mobility of high mol wt serum ALP was greatly reduced by incubation with neuraminidase, it seems likely that much of this charge was due to the presence of sialic acid residues.
2. The inactivation curves of both liver and high mol wt serum ALP during treatment with 5 mol/l urea were monoexponential with respect to time and exhibited similar half-lives. No qualitative effect on the structural integrity of the two isoenzymes was observed.

   The heat inactivation curves of high mol wt ALP from serum and bile were both biphasic. Overall, high mol wt ALP was slightly more heat-labile than the liver

isoenzyme.

3. Electrophoresis and Sepharose 6B column chromatography suggested a qualitative and quantitative association between the high mol wt components of ALP, γGT, LAP and 5'NT (all membrane marker enzymes) both in serum and in bile. However, ion-exchange chromatography indicated that these high mol wt components probably did not have a constant composition.
4. Treatment with organic solvents and detergents showed that high mol wt ALP, γGT and LAP from both serum and bile all behaved in a similar fashion. They were inactivated by butan-1-ol, unlike the low mol wt isoenzymes, possibly because they were extracted into the organic layer. Treatment with detergents converted them to smaller forms of identical mol wt but these smaller forms were still larger than the low mol wt isoenzymes usually present in serum and bile.
5. There was qualitative evidence for the association of ALP, γGT and LAP activities with LPX. This association was with the high mol wt components of these enzymes rather than the low mol wt components. There was some semi-quantitative and statistical evidence that sera with large amounts of LPX also had large quantities of enzyme associated with LPX and high activities of high

mol wt ALP. High activities of LPX-associated ALP were also associated with high activities of high mol wt ALP. On the other hand, some patients had high activities of high mol wt ALP in their serum with no detectable LPX.

6. These various lines of evidence taken together suggest that the serum and biliary high mol wt forms of ALP, γGT, LAP and 5'NT, and LPX are very similar entities behaving in a similar fashion during physical and chemical manipulation. There is some evidence for a quantitative as well as a qualitative relationship. Study of further LPX-positive sera might help to confirm this. The results are discussed further in Chapter 7.

CHAPTER 5.

CLINICAL EVALUATION OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE

5.1. AIMS OF STUDY

No assessments of the clinical value of quantitative measurements of high mol wt ALP in relation to other biochemical tests have yet been made. This pilot study therefore had three aims:

1) to assess whether high mol wt ALP measurements alone could yield useful diagnostic information not at present provided by other commonly used tests of liver function.
2) to elucidate its role as part of a group of biochemical tests in the computer assisted diagnosis of liver disease.
3) to gain some insight into the circumstances under which high mol wt ALP is released into the circulation.

5.2. SELECTION OF PATIENTS

72 adult patients with liver disease and raised serum ALP activities (>100 iu/l) were studied. Also included in the investigation as controls were 8 healthy volunteers with normal serum ALP activities and 14 patients who had raised serum ALP activities due to bone disease (Table 5.1.).

TABLE 5.1.

Numbers, age and sex distribution of patients studied in each diagnostic group

Diagnostic Group	Abbreviation used in illustrations	Number of patients			Age Range (years)
		Males	Females	Total	
Normal controls	Normals	5	3	8	25-55
Osteomalacia	Bone disease	9	2	11	24-78
Metastatic bone disease		2	1	3	19-65
Serum hepatitis	Acute hep.	2	4	6	19-29
Infectious hepatitis		2	0	2	33-34
Chronic active hepatitis	C.A.H.	1	4	5	30-84
Primary biliary cirrhosis	P.B.C.	0	7	7	38-69
Alcoholic cirrhosis	Alc. cirr.	4	3	7	52-64
Carcinoma of head of pancreas	Ca. pancreas	4	1	5	67-76
Cholangiocarcinoma	Chol. ca.	3	1	4	61-68
Gallstone obstruction/ cholecystitis	Gallstones	3	12	15	25-85
Metastatic liver disease	Liver 2^{o}s	11	10	21	32-82

Patients for study were selected by scrutiny of the ALP measurements carried out by a routine clinical chemistry laboratory, by examination of the patient's notes and by discussion with the doctor looking after the patient. Only patients with established diagnoses were admitted to the study. Bone disease was diagnosed radiologically. Diagnosis of serum hepatitis was made if (a) serum levels of bilirubin or alanine aminotransferase or both were raised and (b) hepatitis B surface antigen was detected by radioimmunoassay. Similarly, a diagnosis of infectious hepatitis was made if (a) serum levels of bilirubin or alanine aminotransferase or both were raised and (b) the patient's serum contained a specific IgM antibody to hepatitis A antigen (detected by radioimmunoassay). Samples were taken during the active period of these diseases. Diagnosis of chronic active hepatitis, alcoholic cirrhosis, primary biliary cirrhosis and cholangiocarcinoma were almost invariably proven by biopsy. Carcinoma of the head of the pancreas was demonstrated at laparotomy. Metastatic liver disease was usually diagnosed by a combination of liver scanning and biopsy. Problems arose with the group designated 'gallstone obstruction/cholecystitis' (Table 5.1.). In every case, the presence of gallstones was shown either at

operation or radiologically prior to operation, but it was not always possible to be certain whether the raised ALP was due to obstruction by the gallstones, to oedema of the biliary tree or to ascending infection affecting the liver. This uncertainty meant that the diagnostic category of gallstone obstruction/cholecystitis was likely to be heterogeneous. Because the categories of serum and infectious hepatitis were small, seemed to exhibit similar behaviour with regard to the chemical measurements and were clinically alike, they were amalgamated into a single diagnostic category of acute viral hepatitis for subsequent statistical analysis. For similar reasons, osteomalacia and metastatic bone disease were combined into a single diagnostic category of bone disease. Carcinoma of the head of the pancreas and cholangiocarcinoma were combined into a single category of extrahepatic carcinoma causing biliary obstruction for the purposes of discriminant function analysis only.

In general, only one plasma sample was analysed from each subject, with the exception of patients with serum or infectious hepatitis who had serial samples taken at intervals of several days following admission. The serial sampling was continued until the patient was discharged from hospital. Only the first of these samples

was included in the general statistical analysis of results.

5.3. METHODS

5.3.1. Biochemical analyses

Table 5.2. shows the substances analysed in the serum of each patient together with the method used, the coefficient of variation and the reference range. With the exception of total ALP activity which is raised in the presence of both liver and bone pathology, all the other variables were chosen to assess some aspect of liver function. ALP isoenzymes were analysed qualitatively by electrophoresis in each serum to check for possible sources of interference in the ion-exchange method for high mol wt ALP. No unusual isoenzymes were found in the study. Bile acids were not measured owing to limitations in time and facilities available.

5.3.2. Statistical analysis

Standard parametric statistical tests have been used throughout. In order to equalise variances and normalise the data (both requirements being necessary for parametric tests), all measurements, except for plasma albumin, were subjected to logarithmic transformation before statistical analysis. In nearly all cases this rendered the variances within each category homogeneous (Snedecor and Cochran, 1967).

TABLE 5.2.

Methods used for biochemical measurements undertaken in the study

Chemical measurement (Abbreviation used in tables)	Method Reference	Coefficient of variation (%)	Reference Range
High molecular weight alkaline phosphatase (HMW)	Ion-exchange Section 2.9	3.5	~1-10 iu/l ~1-5%
Alkaline phosphatase (ALP)	Bowers and McComb (1966)	2.5	40-100 iu/l
γ-glutamyl transferase (γGT)	Rosalki and Tarlow (1974)	9.0	Males: 10-55 iu/l Females: 5-35 iu/l
Leucine aminopeptidase (LAP)	Szasz (1967)	10.0	~30-80 iu/l
Alanine aminotransferase (ALT)	Henry et al (1960)	2.0	10-40 iu/l
Bilirubin (Bili)	Jendrassik and Grof (1938)	3.5	2-17 μmol/l
Lipoprotein-X (LPX)	Seidel et al (1970)	-	Negative
Albumin (Alb)	Doumas et al (1971)	2.4	36-47 g/l

Different approaches have been advocated for computer assisted diagnosis of liver disease. Bayesian probability techniques suffer the twin disadvantages of being based on presence/absence criteria which do not make full use of continuous laboratory data, and of requiring knowledge of incidence rates of diseases, not only in the general population but also in the patients likely to present to a particular clinic. Cluster analysis is largely useful for identifying previously unsuspected groups in unclassified data and is therefore more of a research tool than a diagnostic process. For the purpose of diagnosis based on biochemical data, discriminant function analysis is the most suitable approach (Goldberg and Ellis, 1978). Various authors have combined batteries of liver function tests into discriminant functions for the diagnosis of liver disease (Fellingham and Mekel, 1966; Winkel et al, 1975; Sherr, 1977; Goldberg and Ellis, 1978). This formalises the intuitive approach of clinicians in weighing up the probabilities of the different diagnostic alternatives.

Discriminant analysis is a multivariate technique for studying the extent to which different populations overlap one another or diverge from one another (Snedecor and Cochran, 1967). In the present context it was used

to discover which measurements were most effective in distinguishing between the different diagnostic categories, how these measurements could best be combined to form a discriminant function and how successful this discriminant function was in making the distinction.

For discriminant function analysis, a standard computer program was used (program BMDP7M from the Health Sciences Computing Facility, University of California, Los Angeles). This analysis finds linear combinations of the biochemical variables which most clearly distinguish groups (of diseases) from one another. For each variable, a weighting factor is calculated such that, when the weighted biochemical measurements for each patient are added together, the resulting sums for each patient in a particular group or disease category are similar to one another and differ as much as possible from those for patients in other groups. Depending on the number of groups to be distinguished, a number of discriminant functions may be required before optimal separation in one or more dimensions can be achieved. Variables which do not contribute independently and significantly to the separation of groups are omitted from the discriminant function.

A number of factors affect the tests incorporated

into discriminant functions. Firstly, in small samples as in this pilot study, chance may play a considerable role in the selection of discriminants. This can only be eliminated by a much larger study. Secondly, obviously the choice of liver diseases selected for comparison will affect the discriminants. Thirdly, the overall choice of liver function tests, all of which are interrelated and are more or less correlated with one another, will affect which of the tests are taken as the discriminants. This means that an apparently poorly discriminating test considered singly may be the second discriminant to be entered in a function, simply because all the other apparently more discriminating tests are correlated with the first discriminant to be entered.

Since in this study the discriminant functions obtained could not be evaluated prospectively, a cross-validation procedure was carried out. This involves assignment of each case to the disease category to which it has the highest probability of belonging on the basis of the discriminant functions, having first removed the contribution of that particular case to the derivation of the discriminant functions. Such prognostic classification of patients into the different diagnostic categories may or may not take into account the incidence of each disease

in the general population. I have avoided incorporation of such a priori diagnostic probabilities into the cross-validation procedure because (a) this would weight the classification too heavily in favour of normal controls, only a small proportion of whom are likely to present to the clinician and (b) the relative proportions of the different diagnostic groups at presentation will vary from hospital to hospital and from clinic to clinic and would not therefore have general applicability. It was therefore assumed during cross-validation that the a priori probabilities for the occurrence of each disease in the sampled population were equal and that the observed differences in sample size between the various categories were due solely to sampling differences. The derived classification was then compared with the true classification in order to test the accuracy of the discriminant functions in distinguishing diagnostic categories.

5.4. HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE AS A SINGLE TEST

The results of all the biochemical measurements are presented in Fig 5.1. and Table 5.3. but attention will be primarily focussed on ALP and high mol wt ALP. ALP activities in all the patients studied were, by selection, abnormal. A high degree of overlap occurred between the

Figure 5.1. Results of biochemical measurements in different diagnostic categories. Each data point represents one patient.

(a) Total ALP

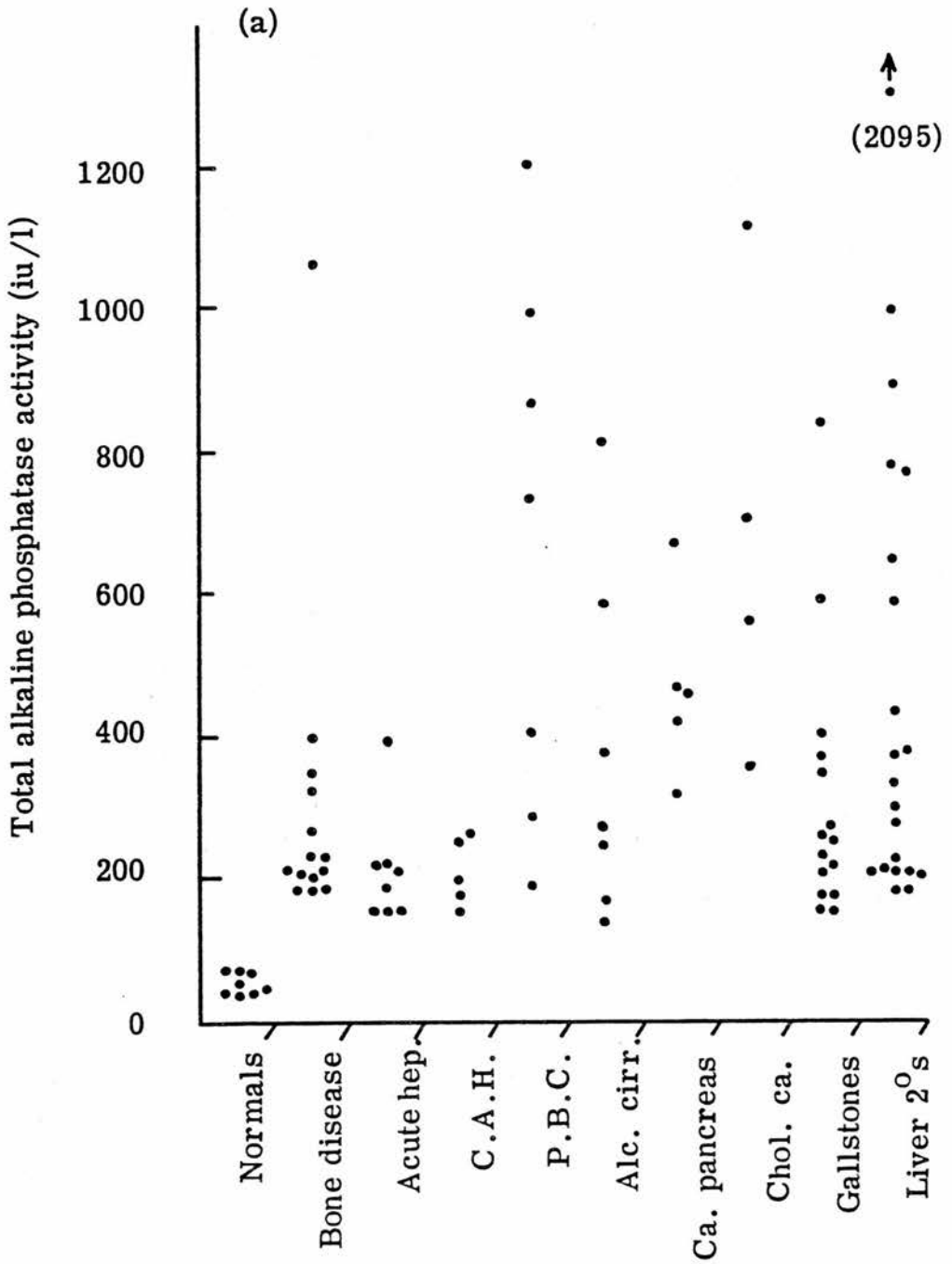


Figure 5.1. (continued)

(b) High mol wt ALP (as iu/l)

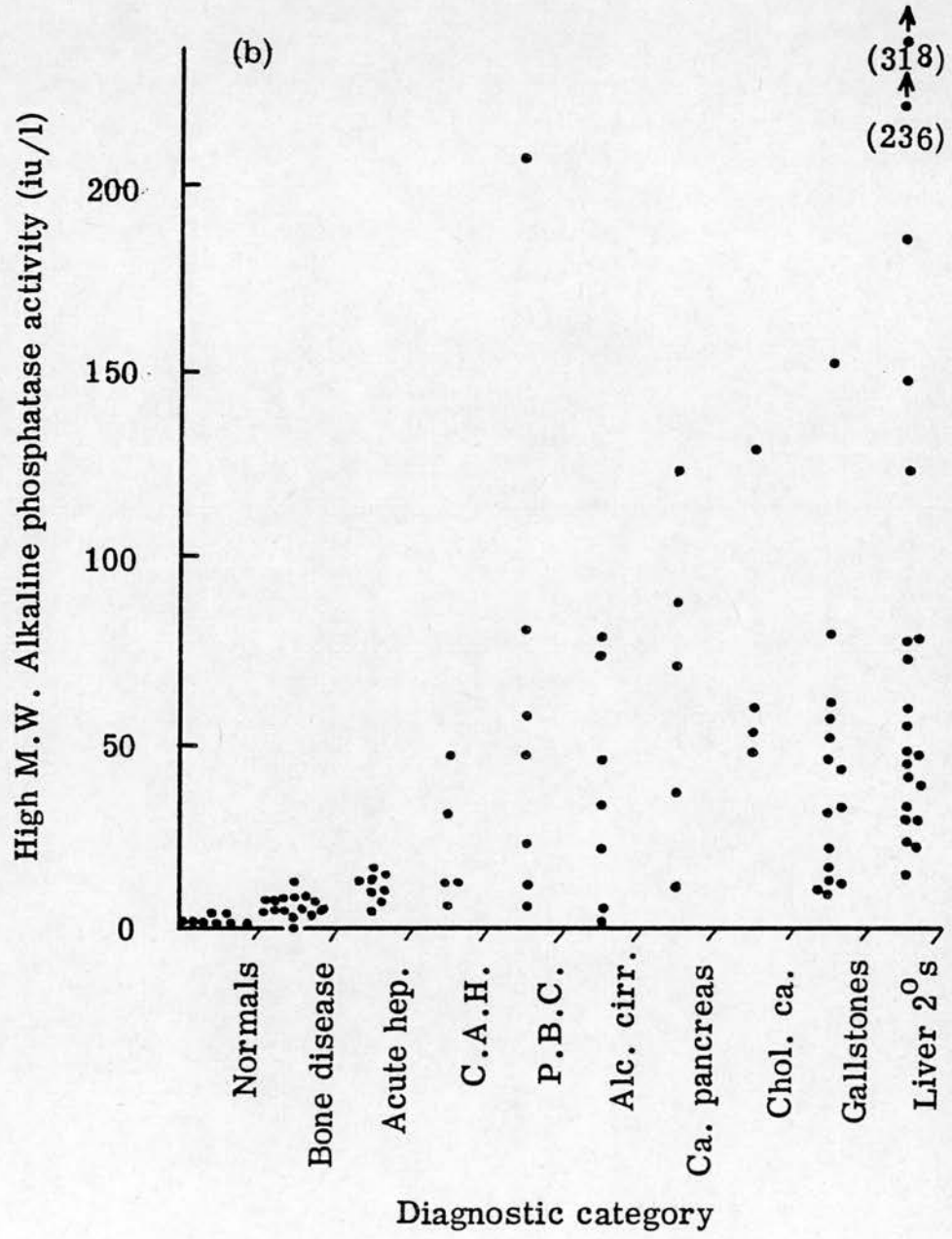


Figure 5.1. (continued)

(c) High mol wt ALP (as percentage of total ALP)

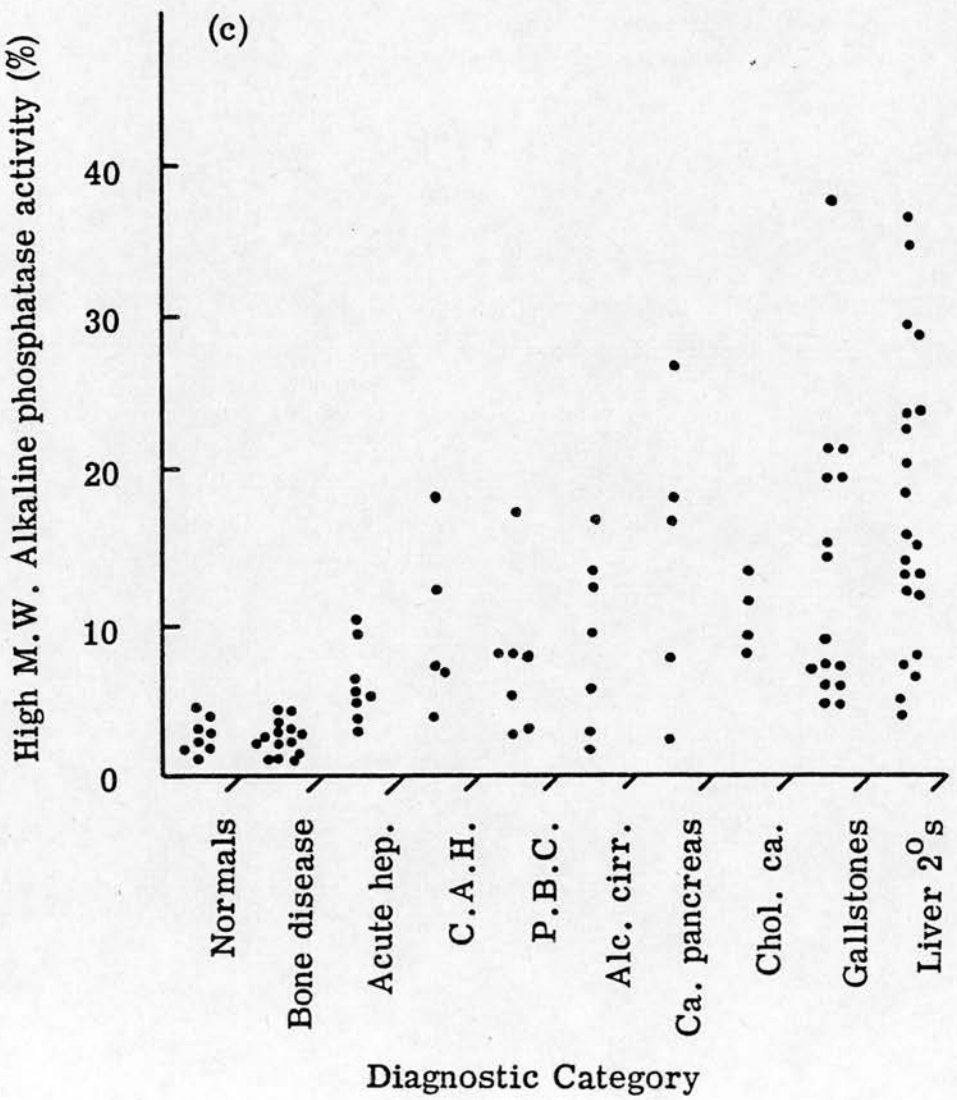


Figure 5.1. (continued).

(d) Bilirubin

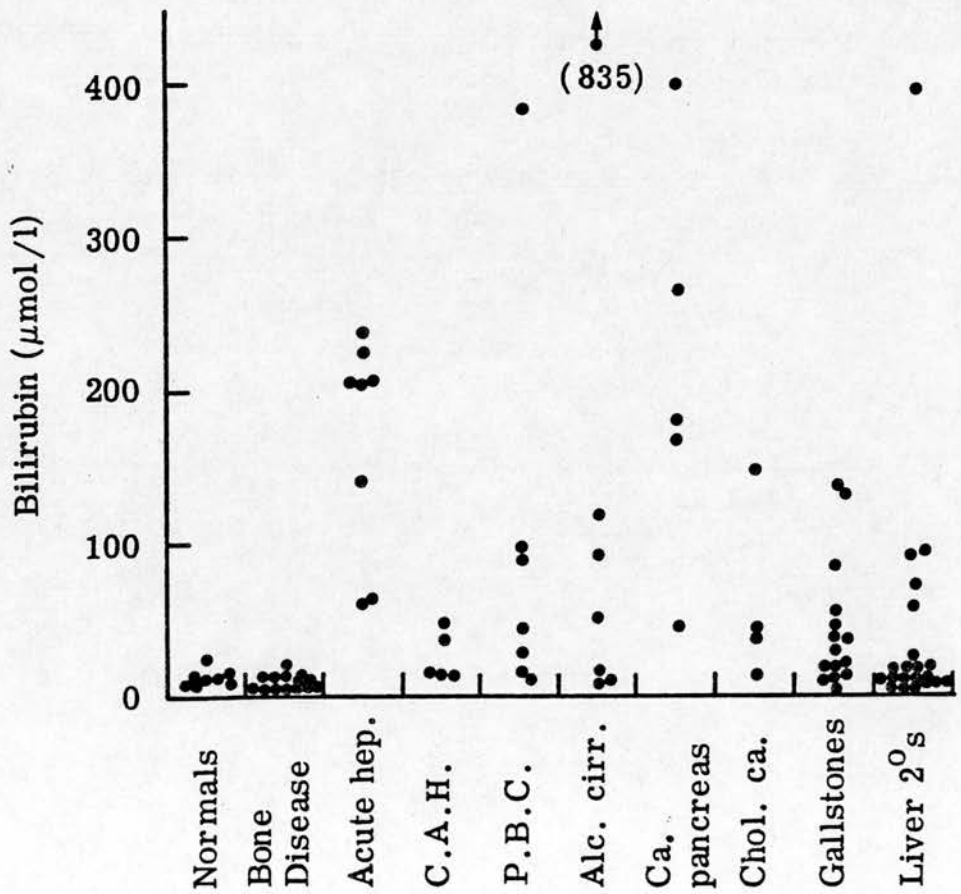


Figure 5.1. (continued).

(e) Alanine aminotransferase

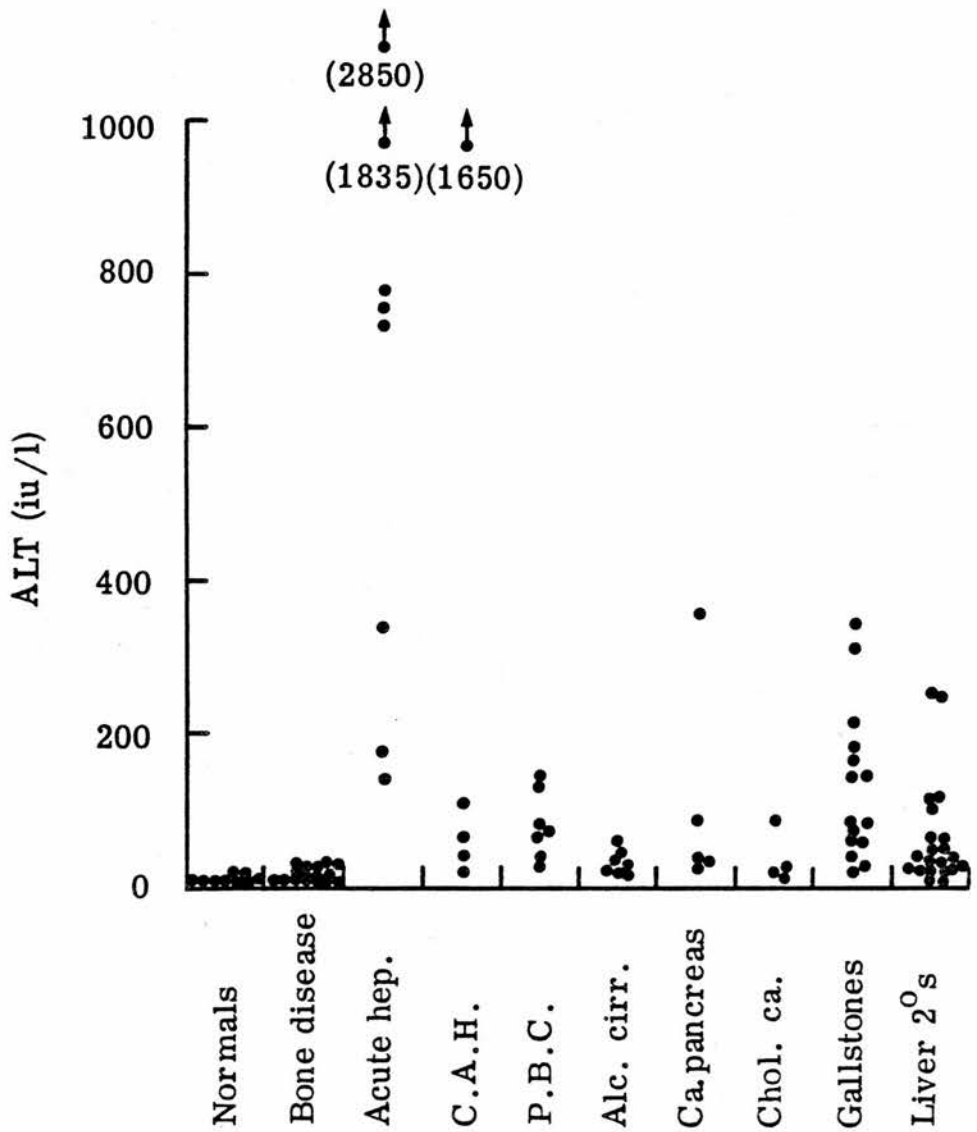


Figure 5.1. (continued).

(f) γGlutamyl transferase

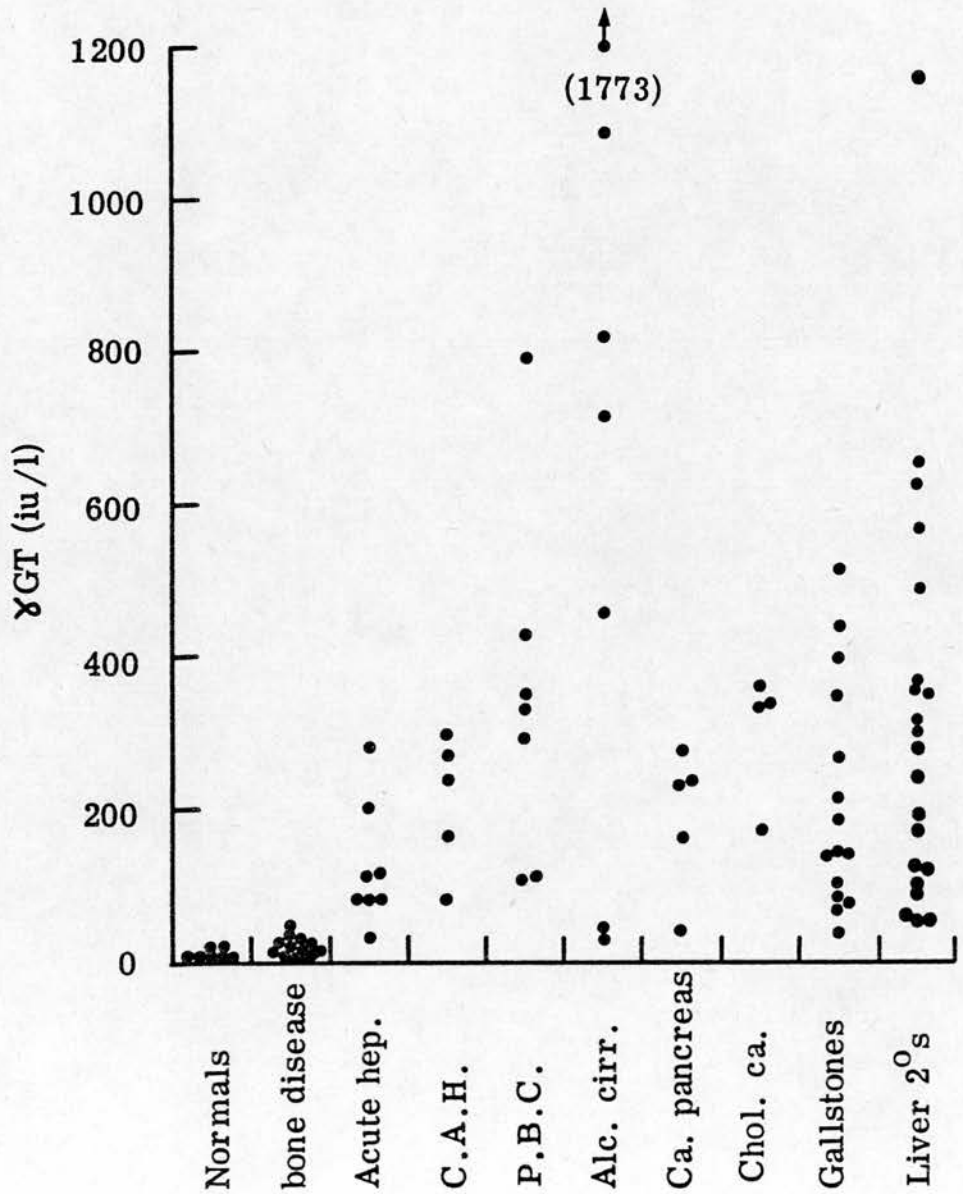


Figure 5.1. (continued).

(g) Leucine aminopeptidase

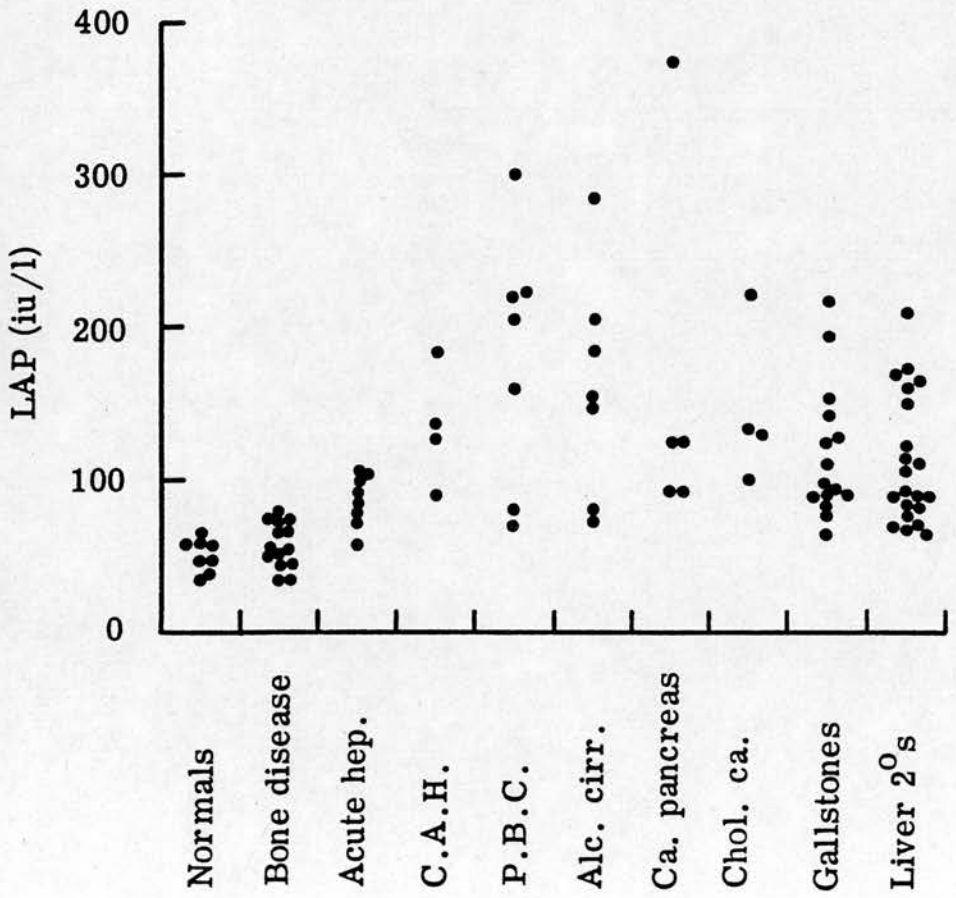


Figure 5.1. (continued).

(h) Albumin

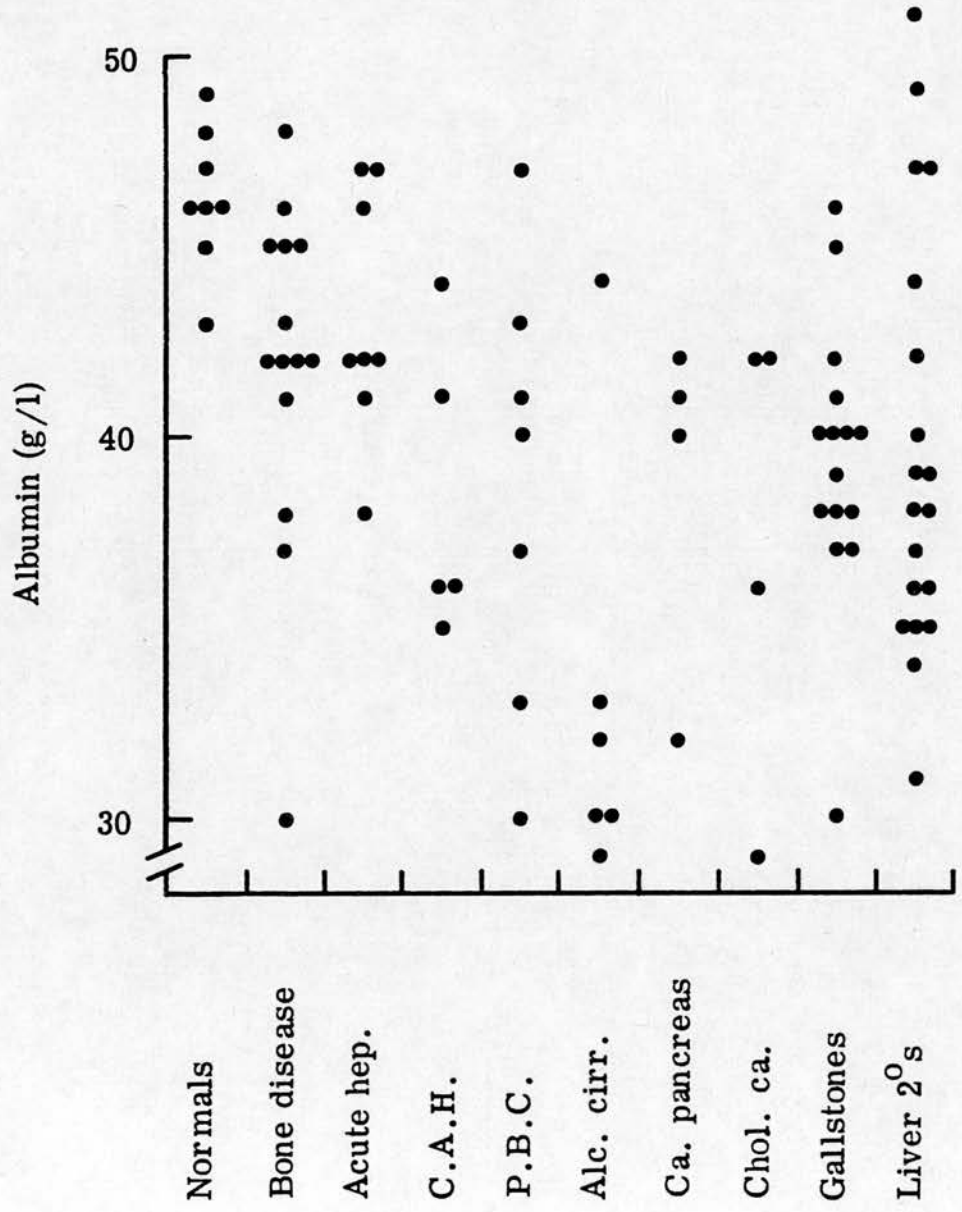


Figure 5.1. (continued).

(i) Lipoprotein X

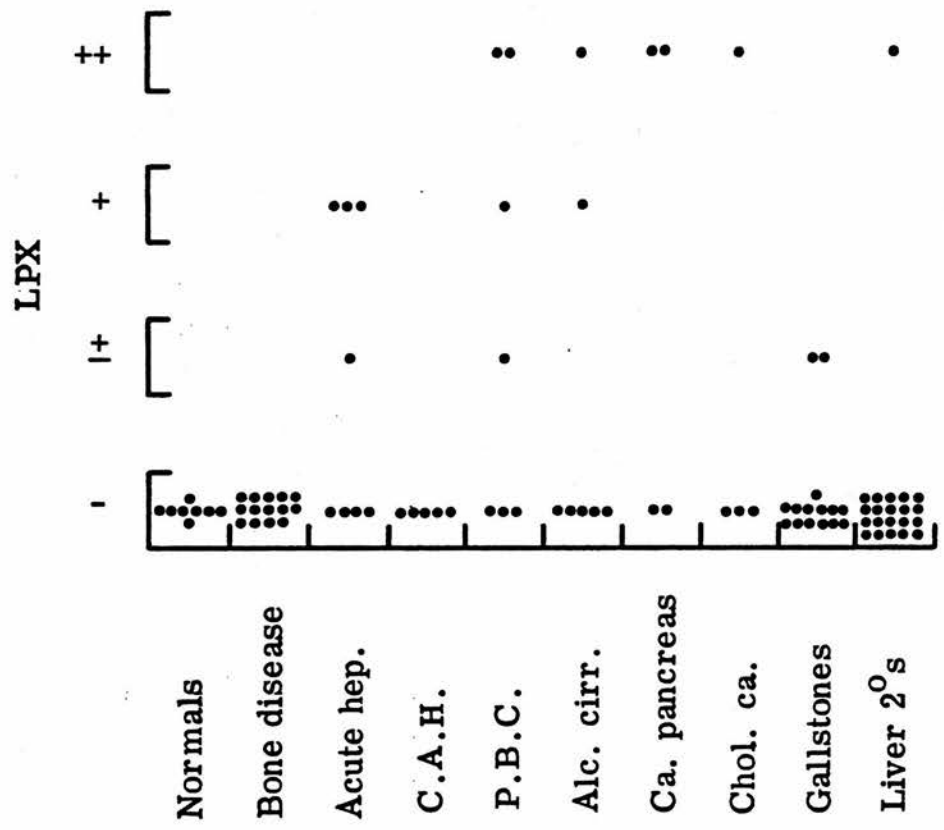


Figure 5.1. (continued).

(j) ALP isoenzymes

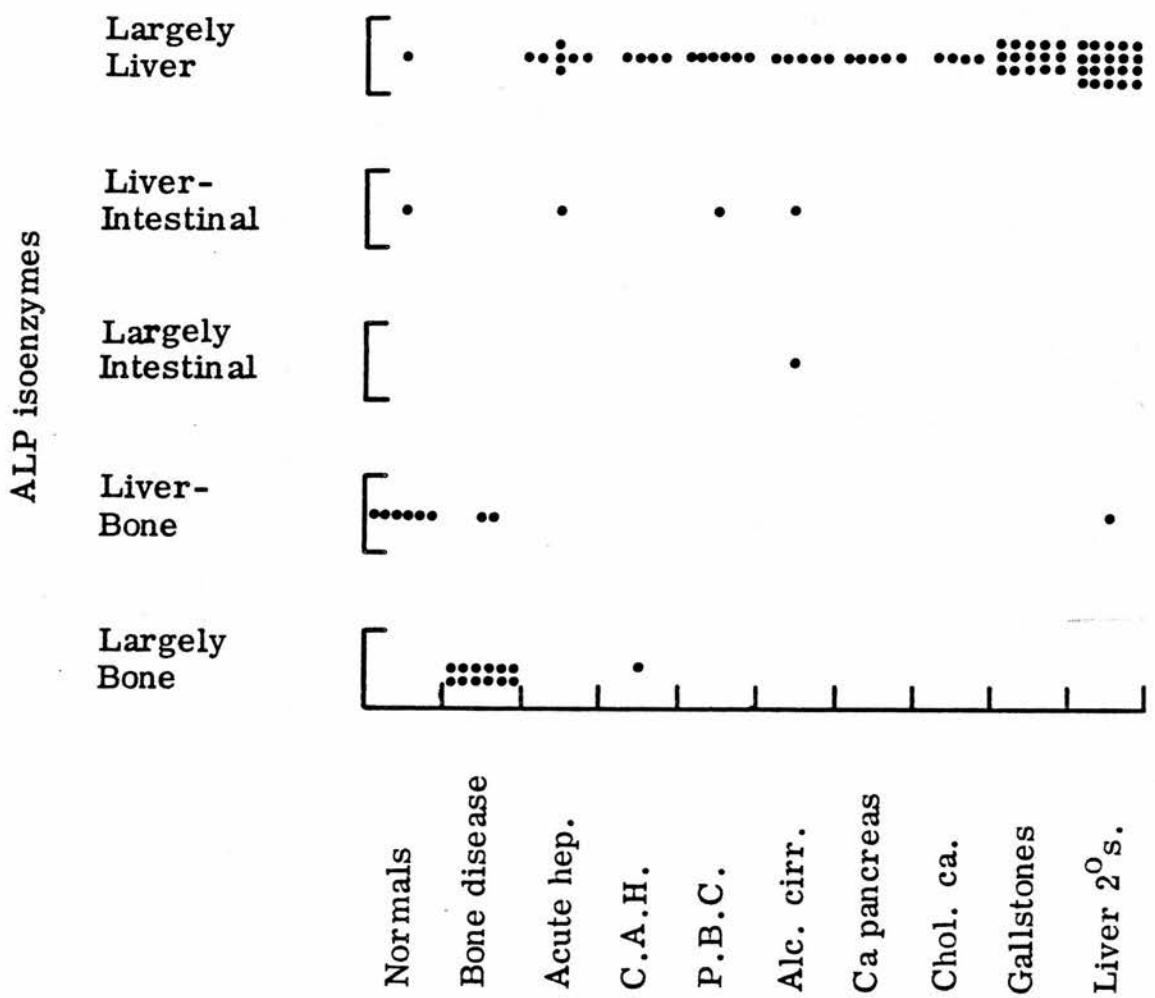


TABLE 5.3.

Geometric means, ranges and frequencies of abnormal results for biochemical tests used in the diagnosis of liver disease

Parameter	Bili (μmol/l)	ALT (iu/l)	LAP (iu/l)	γGT (iu/l)	Alb (g/l)	LPX	ALP (iu/l)	HMW (iu/l)	HMW (%)
Normal controls									
Geometric mean*	11	13	50	11	46	-	50	1	2.2
Range	7-15	10-21	36-64	4-20	43-49	-	33-69	1-2	1.1-4.3
Frequency of abnormal results	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
Bone disease									
Geometric mean*	7	15	56	19	42	-	262	6	2.1
Range	4-20	10-31	35-78	7-47	30-48	-	182-1060	2-13	0.8-4.1
Frequency of abnormal results	1/14	0/14	0/14	1/14	1/14	0/14	14/14	1/14	0/14
Acute hepatitis									
Geometric mean*	151	610	84	107	43	-	201	10	5.2
Range	61-238	140-2850	58-105	84-278	38-47	-	152-392	5-16	2.5-10.0
Frequency of abnormal results	8/8	8/8	5/8	8/8	0/8	3/8	8/8	4/8	4/8
Chronic active hepatitis									
Geometric mean*	22	101	131	192	38	-	203	17	8.4
Range	13-47	20-1650	89-186	84-298	35-44	-	157-258	6-46	3.9-18.3
Frequency of abnormal results	2/5	4/5	5/5	5/5	1/5	0/5	5/5	4/5	4/5
Primary biliary cirrhosis									
Geometric mean*	49	71	161	284	39	-	556	35	6.3
Range	11-385	29-147	71-300	109-789	30-47	-	191-1200	6-207	2.8-17.3
Frequency of abnormal results	5/7	5/7	6/7	7/7	2/7	4/7	7/7	6/7	5/7

TABLE 5.3. (Continued)

	Bili (μmol/l)	ALT (iu/l)	LAP (iu/l)	γGT (iu/l)	Alb (g/l)	LPX	ALP (iu/l)	HMW (iu/l)	HMW (%)
Alcoholic cirrhosis									
Geometric mean*	47	30	147	352	33	-	309	22	7.1
Range	8-835	13-58	73-286	30-1773	29-44	-	139-815	3-78	2.1-16.7
Frequency of abnormal results	4/7	2/7	5/7	6/7	5/6	2/7	7/7	5/7	5/7
Carcinoma of head of pancreas									
Geometric mean*	171	61	137	159	39	-	453	50	10.9
Range	46-400	23-360	92-372	43-373	32-42	-	320-670	11-122	2.4-27.1
Frequency of abnormal results	5/5	2/5	5/5	4/5	1/5	3/5	5/5	5/5	4/5
Cholangiocarcinoma									
Geometric mean*	43	26	140	292	37	-	628	65	10.4
Range	13-147	10-87	100-222	176-362	29-42	-	353-1115	51-128	8.2-13.4
Frequency of abnormal results	3/4	1/4	4/4	4/4	1/4	1/4	4/4	4/4	4/4
Gallstones/Cholecystitis									
Geometric mean*	28	98	111	167	39	-	273	30	11.1
Range	3-136	21-345	65-218	41-521	30-46	-	158-835	9-151	4.9-37.7
Frequency of abnormal results	11/15	13/15	13/15	15/15	1/15	2/15	15/15	13/15	14/15
Metastatic liver disease									
Geometric mean*	19	43	105	228	40	-	383	57	15.0
Range	5-395	10-253	64-211	55-1160	31-51	-	181-2095	14-318	4.4-36.5
Frequency of abnormal results	9/21	9/21	16/21	21/21	5/21	1/21	21/21	21/21	20/21

* Statistical analysis carried out on data previously subjected to logarithmic transformation, with the exception of albumin. Figures given in the table have been converted back into an arithmetic scale.

results from patients with a wide variety of liver diseases and patients with bone disease (Fig 5.1.(a), Table 5.3.).

High mol wt ALP activity was expressed both in iu/l and as a percentage of total ALP activity. In order of ascending high mol wt ALP activity, the following groups had means which were significantly different from one another ($p < 0.05$) using the Q method for differences between means (Snedecor and Cochran, 1967) (Fig 5.1.(b), Table 5.3.):

1. Normal controls
2. Bone disease
3. Acute hepatitis, chronic active hepatitis, alcoholic cirrhosis
4. Primary biliary cirrhosis, gallstones/cholecystitis
5. Carcinoma of the head of the pancreas, cholangiocarcinoma and metastatic liver disease.

High mol wt ALP activities were therefore higher (1) in liver disease than in bone disease and normal controls (2) in obstructive lesions than in non-obstructive lesions and (3) in patients with obstruction due to carcinoma in the liver or its vicinity than in non-neoplastic forms of obstruction.

When the results were expressed as a percentage of total ALP activity the overall pattern was similar

(Fig 5.1.(c), Table 5.3.). However, the difference between the normal controls and patients with bone disease was no longer apparent. On the other hand, the discrimination between metastatic liver disease and other forms of liver disease was better than when the results were reported in units of activity: t-test analysis showed that, of all the chemical measurements, the percentage activity of high mol wt ALP distinguished metastatic from all other forms of liver disease most effectively ($p < 0.005$, all other chemical measurements non-significant). Nevertheless the overlap between groups was so great that only when a very high percentage high mol wt ALP is found (arbitrarily defined as over 25%) could such a result be considered a strong pointer to a diagnosis of liver metastases, in the absence of any other indicator. However, if a patient were already known to have a primary carcinoma suspicion of liver metastases might be aroused by a considerably lower level of high mol wt ALP.

Comparison with previous findings. Many of the methods used to demonstrate the presence of high mol wt ALP in liver disease have been qualitative electrophoretic techniques which can only be made semi-quantitative by means of densitometry or elution. On a purely qualitative basis, Burlina and Galzigna (1976) found that the $\alpha_1$globulin ALP

(equivalent to high mol wt ALP) on cellulose acetate electrophoresis appeared in all cases of obstructive jaundice, in some cases of cirrhosis but in virtually no cases of viral hepatitis or bone disease and in no normal controls. Semi-quantitation of the α_1 globulin band by densitometry (Rhone et al, 1973) gave the highest levels in liver metastases with intermediate levels in cirrhosis and relatively low levels in gallstone obstruction/ cholecystitis and acute hepatitis. Semi-quantitation of the origin bands in starch gel (Kowlessar, Haeffner and Riley, 1961) and polyacrylamide gel (Price and Sammons, 1976) by elution showed similar high levels in obstructive jaundice and metastatic liver disease but lower levels in acute hepatitis and undetectable amounts in normal controls and patients with bone disease. All these results are in conformity with the present findings.

Two quantitative studies on small numbers of patients, based on Sephadex G200 gel filtration (Fennelly et al, 1969; Jennings et al, 1970) have produced an essentially similar distribution of results, the highest percentage of high mol wt ALP being seen in metastatic liver disease. The actual levels tended to be slightly higher than I have observed, possibly because the void volume peak on Sephadex G200 chromatography may contain

non-specific βlipoprotein-associated ALP (see section 2.10 and 4.8) and therefore overestimates high mol wt ALP. In none of these clinical studies was measurement of any biochemical variable other than total and high mol wt ALP undertaken.

The observation in the present study that high mol wt ALP has a higher activity in bone disease than in normal controls, but forms a similar percentage of the total ALP in each category is in agreement with the findings of Fennelly et al (1969). This could have been due to carry-over in the method although investigations could find no evidence for this (section 2.9.2.) and high mol wt ALP, measured by the ion-exchange method, showed no significant correlation with total ALP in bone disease as might have been expected if carry-over had occurred. Alternatively, some of the patients with bone disease (e.g. bone metastases, osteomalacia) may have had a slight degree of co-existing liver disease which had been undetected clinically or biochemically.

5.5. LIPOPROTEIN X

LPX was negative in normals and in patients with bone disease but was at least transiently positive in some cases of acute hepatitis, possibly reflecting a degree of biliary stasis in these patients. It was most often

positive in primary biliary cirrhosis, but in general the test appeared to lack sensitivity. This may have been related to the use of an antiserum of fairly low titre since LPX has previously been found to be positive in over 90% of patients with histological evidence of cholestasis and, conversely, negative in a similar percentage of patients with no histological evidence of cholestasis (Fellin et al, 1978). Its correlation with high mol wt ALP is presented in section 4.8.3.

5.6. ROLE OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE IN THE COMPUTER DIAGNOSIS OF LIVER DISEASE

5.6.1. Non-necrotic liver diseases combined

Inspection of Fig 5.1. and Table 5.3. shows that individual tests exhibit overlap between most disease categories, particularly within those categories of patients with liver disease. The tests were therefore combined into discriminant functions which were initially designed to answer three questions:

1. Does the patient have liver disease or is he healthy?
2. Where there is a raised serum ALP activity, is this due to liver or bone disease?
3. If the patient has liver disease, is it of a necrotic type (acute hepatitis) or a non-necrotic type?

For these purposes, four broad categories of disease

were defined:

a) normal individuals

b) bone disease

c) acute hepatitis

d) all other forms of liver disease excluding acute hepatitis.

Table 5.4. shows the tests which contribute to the discriminant for each possible pair of diagnostic categories, together with their weighting factors (see section 5.3.2.) and the percentage of correct classifications achieved by the cross-validation procedure. The discrimination is also shown diagrammatically in Fig 5.2. High mol wt ALP is present in 4 of the 6 pairs as one of the discriminants. It was only excluded from a fifth function because its F value was marginally lower than the F value for LAP. Once the latter had been entered into the discriminant the F value of high mol wt ALP fell to insignificant levels because it was correlated with LAP (see Table 5.4.).

It is clear from Table 5.4 and Fig 5.2. that the first two of the questions posed above can almost invariably be correctly answered by means of discriminant function analysis. The third could be answered with 91% accuracy. Although high mol wt ALP was one of the discriminators in deciding whether a raised ALP was of liver or bone origin,

TABLE 5.4.

The discriminants (in order of entry into discriminant function), weighting factors and accuracy of cross-validation for each pair of diagnostic categories

Diagnostic categories compared	Discriminants[a] (weighting factor)	Number correctly classified	Percentage correctly classified
Normals vs Bone disease	ALP; HMW (0.98)	7/7 14/14	100
Normals vs Acute hepatitis	HMW; Bili (0.72)	7/7 8/8	100
Normals vs Non-necrotic liver disease	HMW; ALT (0.68); γGT (0.72); Alb (-0.053)	7/7 58/59	99
Bone disease vs Acute hepatitis	Bili; ALT (0.50)	14/14 8/8	100
Bone disease vs Non-necrotic liver disease	γGT; ALP (-1.90); HMW (1.23); Bili (0.50)	14/14 57/59	97
Acute hepatitis vs Non-necrotic liver disease	ALT; LAP[b] (-1.20)	7/8 54/59	91

a All discriminants are in log. form with the exception of albumin. The weighting factor of the best single discriminator in each case has been set to unity.

b F values of HMW and LAP were virtually identical but once LAP had been entered into the discriminant, the F value of HMW fell to an insignificant level. For correlation of HMW with LAP see Table 5.8.

Figure 5.2. Discriminant functions (see Table 5.4.) plotted for each pair of diagnostic groups as shown. Each point represents one patient.

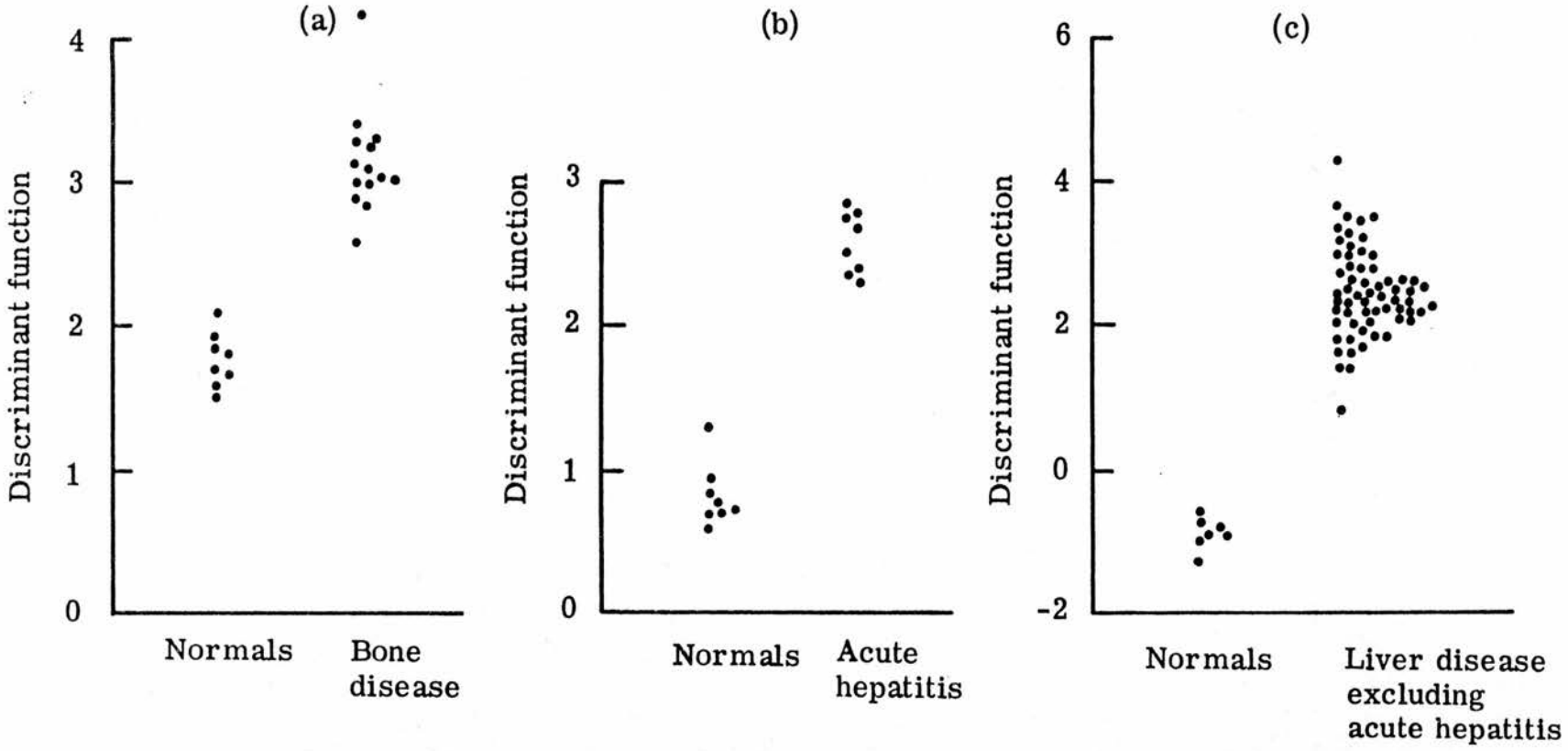
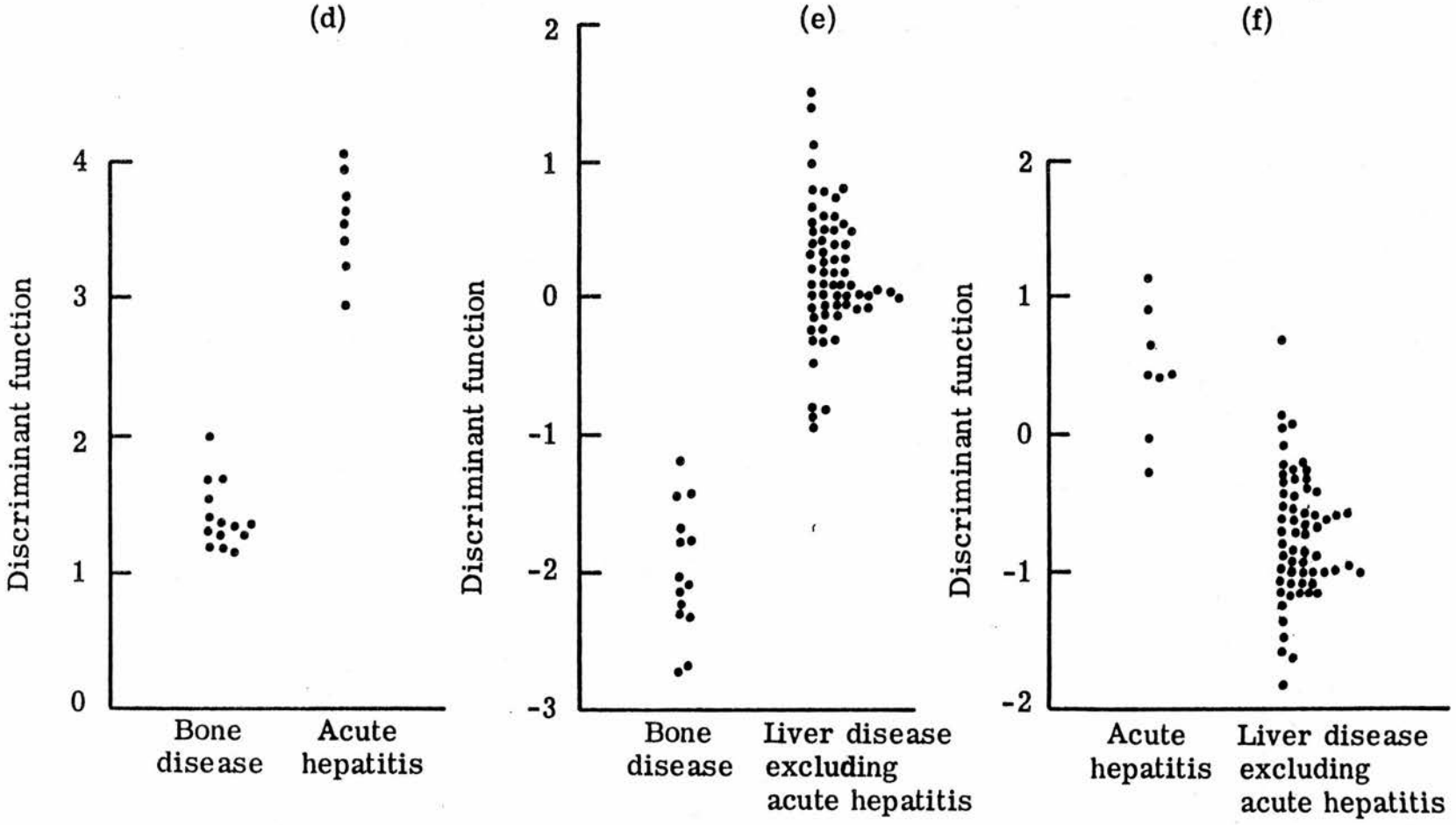


Figure 5.2. (continued).



bilirubin and alanine aminotransferase (for acute hepatitis), γGT (for non-necrotic liver disease) or iso-enzyme separation techniques are simpler tests which are more effective. However, high mol wt ALP appeared to be the best single discriminator in deciding whether or not a patient had liver disease. In this respect, it appeared to be superior even to γGT.

In an attempt to distinguish all four categories simultaneously, it was found that all the variables except LAP and albumin contributed to a set of three discriminant functions (Table 5.5.). The first two of these plotted against each other (Fig 5.3.) revealed four distinct clusters. Cross-validation led to a correct classification of 89% of the patients among the four groups. All except one of the incorrect classifications occurred in the group of non-necrotic liver diseases, some of whom were incorrectly assigned to the acute hepatitis group.

5.6.2. <u>All liver diseases considered separately</u>

Having established that a patient has liver disease, the next step is to identify the correct diagnosis. An attempt to distinguish all 7 liver diseases simultaneously led to a correct classification of only 39% of the patients among the seven groups.

The liver diseases were therefore compared in pairs.

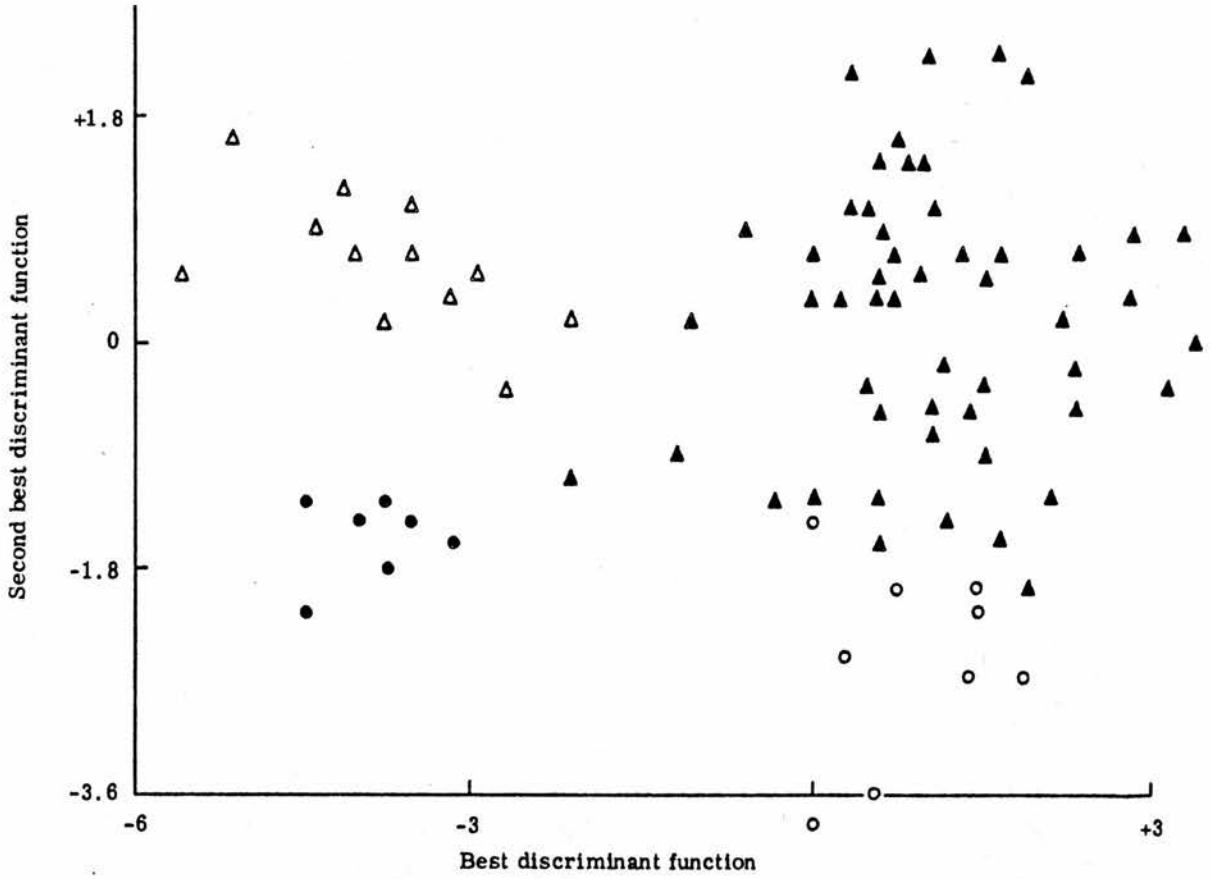
TABLE 5.5.

The discriminants (in order of entry into discriminant function) and weighting factors used to achieve maximum discrimination between a) normals b) bone disease c) acute hepatitis and d) non-necrotic liver disease

Discriminant function	Weighting factors of variables entered*				
	ɣGT	ALT	ALP	HMW	Bili
1	2.0	0.8	- 2.8	2.5	0.8
2	0.2	- 1.0	2.8	0.2	- 1.1
3	1.3	- 2.0	- 4.6	1.2	0.6

* The variables are expressed in logarithmic form.

Figure 5.3. Second discriminant function plotted against the first in the overall discrimination between four diagnostic groups (see text and Table 5.5.). ● normal controls; △ bone disease; O acute hepatitis; ▲ non-necrotic liver disease.



The variables (together with their weighting factors) contributing to the single discriminant function for each pair are shown in Table 5.6. Usually, optimal separation of the pairs was achieved by the use of only two discriminants since the addition of further discriminants did not improve diagnostic accuracy. However, occasionally only one discriminant contributed and sometimes 3 were needed. In three of the pairs, no combination of the variables could be found to discriminate. Cross-validation of the discriminant functions led to accurate classification of most patients (Table 5.7.).

High mol wt ALP contributed to the discriminant functions in 6 out of the possible 21 pairs i.e. it contributed as frequently as total ALP and LAP although less frequently than alanine aminotransferase. In particular, it appeared most frequently in the discriminants distinguishing liver metastases from other liver diseases.

As far as tests other than high mol wt ALP are concerned these conformed to established patterns of behaviour in liver disease (Tables 5.3. and 5.6.). Alanine aminotransferase was particularly useful in the discrimination of acute hepatitis from other liver diseases. Bilirubin, although high in acute hepatitis, was not helpful in distinguishing between different types of liver diseases.

TABLE 5.6.

Variables contributing to the discriminant functions in the distinction of pairs of liver diseases. The weighting factors for the log-transformed discriminants are shown in brackets

	C.A.H.	P.B.C.	Alc.cirr.	Ex.hep.[a] ca.	Gallstones	Liver 2^{o}s
Acute hep.	Bili(3.8) ɣGT(-2.8)	ALT(4.9) ALP(-6.9)	ALT(5.5) LAP(-12.0)	ALP(-11.0) ALT(2.8)	ALT(3.0) Bili(2.7) LAP(-7.5)	ALT(2.9) HMW(-3.2)
C.A.H		ALP(-24.2) HMW(10.2)	ALT	ALP(-18.5) Bili(-4.2)	b	HMW(-5.1) LAP(7.5)
P.B.C.			ALT(10.1) ɣGT(-7.6) ALP(7.4)	b	ALP(11.3) HMW(-4.7)	LAP(10.4) HMW(-4.0)
Alc.cirr.				b	Alb(-0.4) LAP(9.3)	Alb(-0.2) LAP(9.2) HMW(-3.1)
Ex.hep.ca.[a]					ALP(7.8) Bili(2.5)	Bili(4.5) ALT(-3.9)
Gallstones						ALT

a Extra-hepatic carcinoma causing biliary obstruction i.e. carcinoma of the head of the pancreas and cholangiocarcinoma.

b No significant discrimination possible between these liver disease categories using these tests.

TABLE 5.7.

Percentage accuracy of cross-validation classification of cases into pairs of liver diseases

	C.A.H.	P.B.C.	Alc.cirr.	Ex.hep. ca.	Gallstones	Liver 2^{o}s
Acute hep.	100	93	100	100	91	96
C.A.H.		82	80	92	b	91
P.B.C.			85	b	77	77
Alc.cirr.				b	81	80
Ex.hep.ca.					87	74
Gallstones						74

[b] No significant discrimination possible between these liver disease categories.

γGT was a sensitive indicator of liver disease (c.f. Szczeklik et al, 1961; Wieme and Demeulenaere, 1970), reaching its highest levels under the influence of induction in alcoholic cirrhosis, but was a poor discriminator between different types of liver disease (c.f. Rutenburg et al, 1963). LAP was a fairly insensitive test and a poor discriminant taken singly but formed the second discriminant in several functions. The high levels in biliary obstruction and relatively low levels in metastatic liver disease are in agreement with the observations of Wieme and Demeulenaere (1970). Albumin was low in alcoholic cirrhosis but was otherwise unhelpful.

5.6.3. Comparison with previous computer assisted diagnostic studies

When liver diseases were compared in pairs, the percentages of patients who were correctly classified by the cross-validation procedure were similar to those found by earlier computer diagnostic studies using different combinations of discriminants (Solberg et al, 1975). The low percentage of correct classification when all the liver diseases were considered simultaneously was also similar to those found by earlier studies (Fellingham and Mekel, 1966; Solberg et al, 1975; Sherr, 1977). Classification accuracy tends to decrease as the number of liver disease categories

under consideration increases. Some previous studies, perhaps for this reason, have tended to group liver diseases in ways that seem clinically incompatible (e.g. liver metastases, liver abscess, typhoid, sarcoidosis, bilharzia, extrahepatic obstruction etc in one group (Fellingham and Mekel, 1966). As illustrated in this example, such a group may include liver diseases which are relatively uncommon in Britain. Despite the drawbacks it was felt in the present study that discriminant function analysis, to be realistic, must incorporate most of the commoner liver diseases in separate categories, as far as possible. Ideally, the discriminant functions obtained would then be combined with probability data based on clinical criteria to achieve optimal diagnostic accuracy.

5.7. CORRELATION BETWEEN HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE AND OTHER LIVER FUNCTION TESTS

The group of patients with secondary carcinoma in the liver was chosen for calculating correlations between the variables because a) this was the largest diagnostic group in the study, b) the group was of particular interest because of the high levels of high mol wt ALP observed and c) it was considered to be invalid to combine all liver diseases in deriving these correlations because the variables might be influenced by different factors in

different diseases e.g. bilirubin in acute hepatitis and extrahepatic obstruction.

Significant correlations (Table 5.8.) were observed between γGT, LAP and ALP, thereby confirming an earlier report (Wieme and Demeulenaere, 1970). In general, high mol wt ALP was less well correlated with the other liver function tests than these were among themselves, with the exception of total ALP. In particular, no relationship at all was observed with alanine aminotransferase, an index of cell necrosis, and the correlation with degree of jaundice was non-significant. There was only a low correlation between high mol wt ALP and total γGT and LAP, indicating that the pathological factors which govern the release of high mol wt ALP into the circulation in metastatic liver disease operate in a different way on the release of total γGT and LAP, both of which may, like ALP, occur largely in low mol wt forms (see Fig 4.6.). For all three enzymes it seems likely that different factors govern the release of the low and high mol wt enzymes into the circulation.

It was not possible in this study to measure high mol wt γGT and LAP in each serum since the ion-exchange assay would have had to be repeated three times with buffers of different ionic strength and this was imprac-

TABLE 5.8(a).

Correlation coefficients between liver function tests measured in 21 patients with metastatic liver disease (data were log-transformed)

	Bili	ALT	LAP	ɣGT	ALP	HMW
Bili		.70	.36	.53	.71	.32
ALT			.28	.41	.43	.08
LAP				.58	.71	.44
ɣGT					.69	.23
ALP						.69
HMW						

TABLE 5.8(b).

Two-tailed probabilities associated with correlation coefficients between liver function tests measured in 21 patients with metastatic liver disease

	Bili	ALT	LAP	ɣGT	ALP	HMW
Bili		<.001	N.S.	<.05	<.001	N S.
ALT			N.S.	N.S.	<.05	N.S.
LAP				<.01	<.001	<.05
ɣGT					<.001	N.S.
ALP						<.001
HMW						

ticable both from the point of view of time available and volume of serum required. Correlations between the high mol wt enzymes alone were therefore not investigated.

5.8. SERIAL MEASUREMENTS IN INDIVIDUAL PATIENTS

Because patients with serum and infectious hepatitis tend to have a well-defined acute illness, these diseases were chosen to investigate the relationship of high mol wt ALP with other variables in individual patients. The following patients were studied (Fig 5.4.).

a) 3 patients with serum hepatitis alone (patients A,B,C)
b) 1 patient with serum hepatitis and chronic cardiac failure (patient D)
c) 2 patients with infectious hepatitis (patients E,F)

On admission all patients were jaundiced to a greater or lesser extent and had raised alanine aminotransferase activities in their serum, although in some cases the activity peak had not yet been reached (Fig 5.4.). The degree of cholestasis at this stage, as indicated by ALP, γGT and LAP activities, was variable but present to some degree. The serum from 2 patients (patients C and F) were even transiently positive for LPX.

The pattern of the biochemical measurements was variable over the course of the disease but some trends could be discerned. Usually, serum bilirubin and certain

Figure 5.4. Serial measurements following admission in 8 patients. Patients A, B, C, serum hepatitis; D, serum hepatitis and chronic cardiac failure (not included in general statistical analyses); E, F, infectious hepatitis; G, carcinoma of the head of the pancreas; H, cholangiocarcinoma.

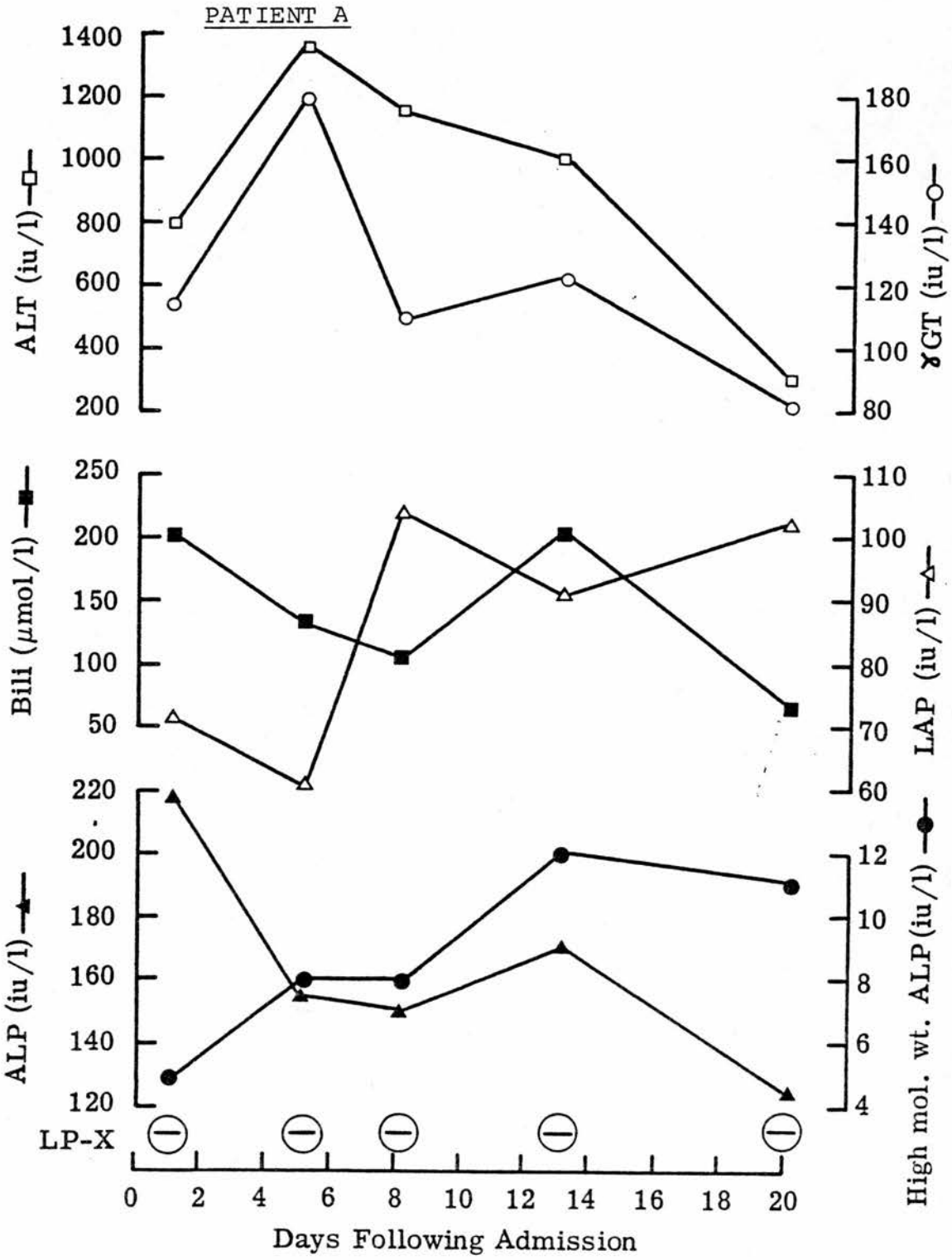


Figure 5.4. (continued).

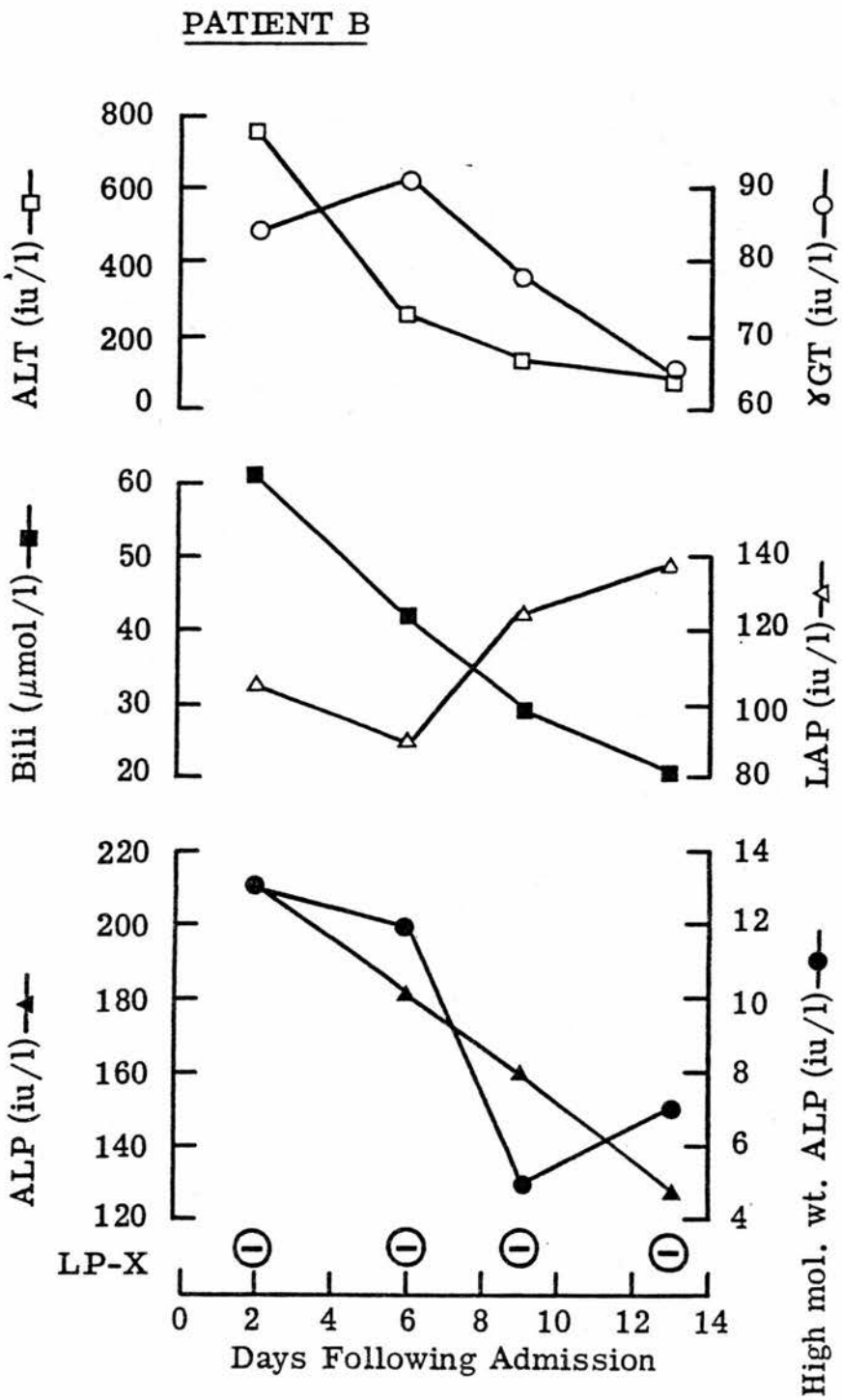


Figure 5.4. (continued).

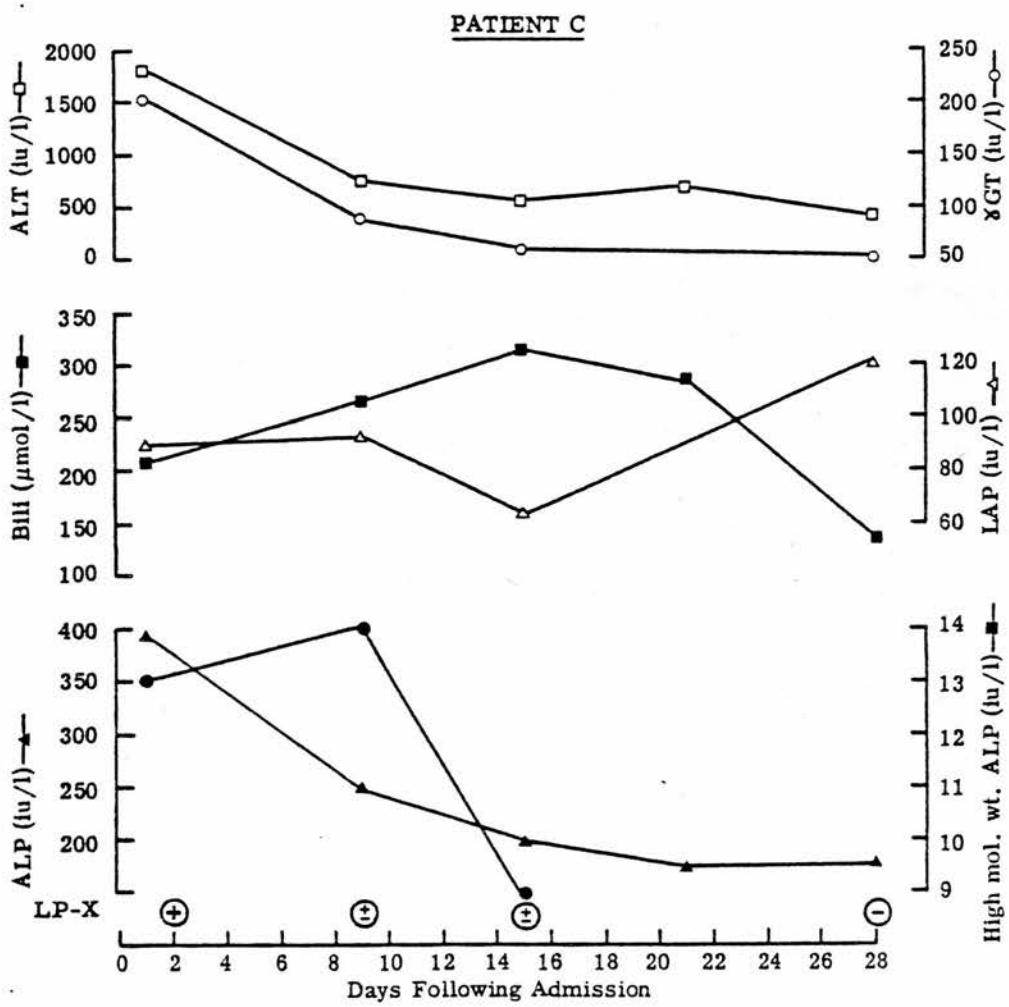


Figure 5.4. (continued).

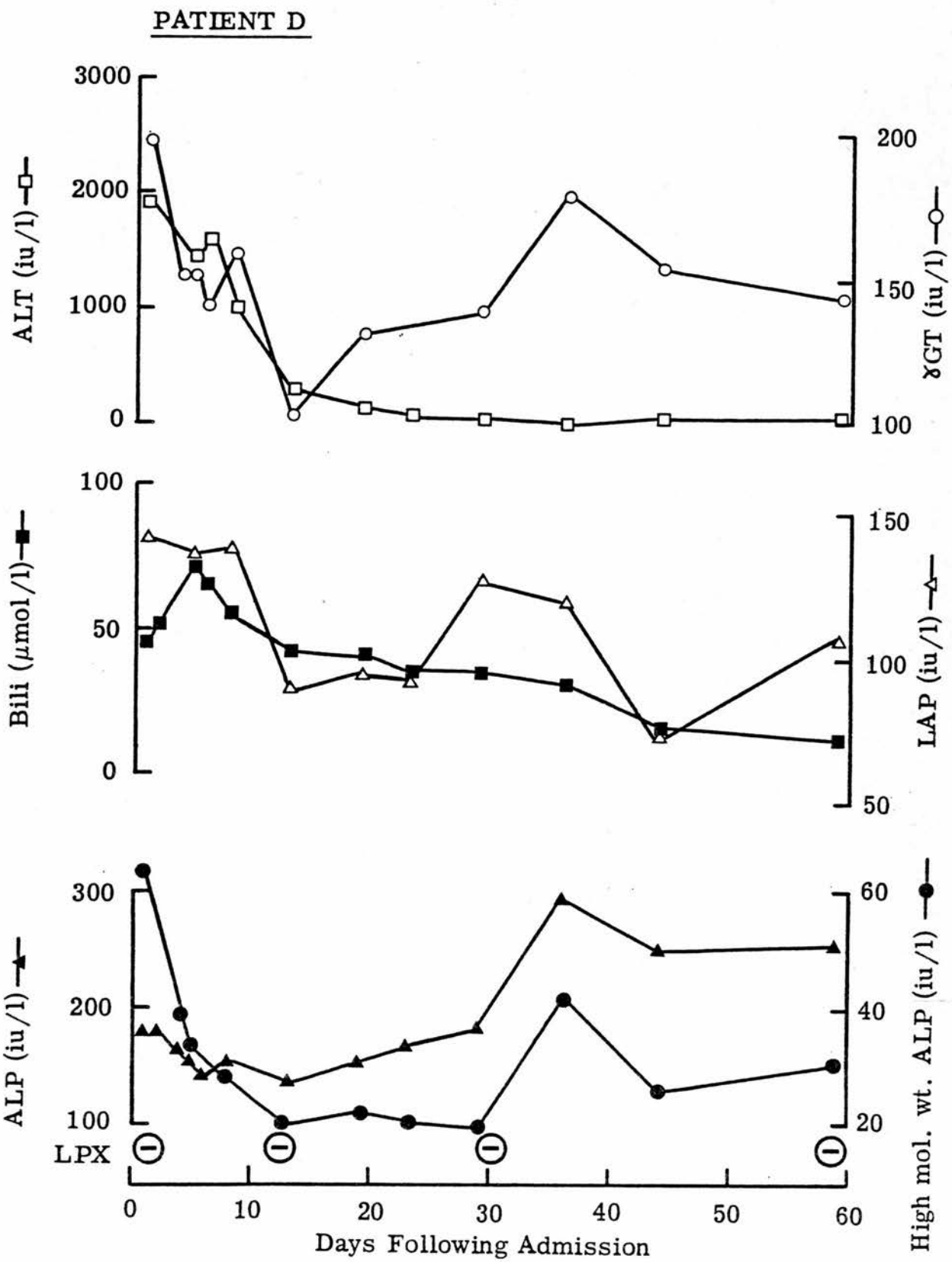


Figure 5.4. (continued).

PATIENT E

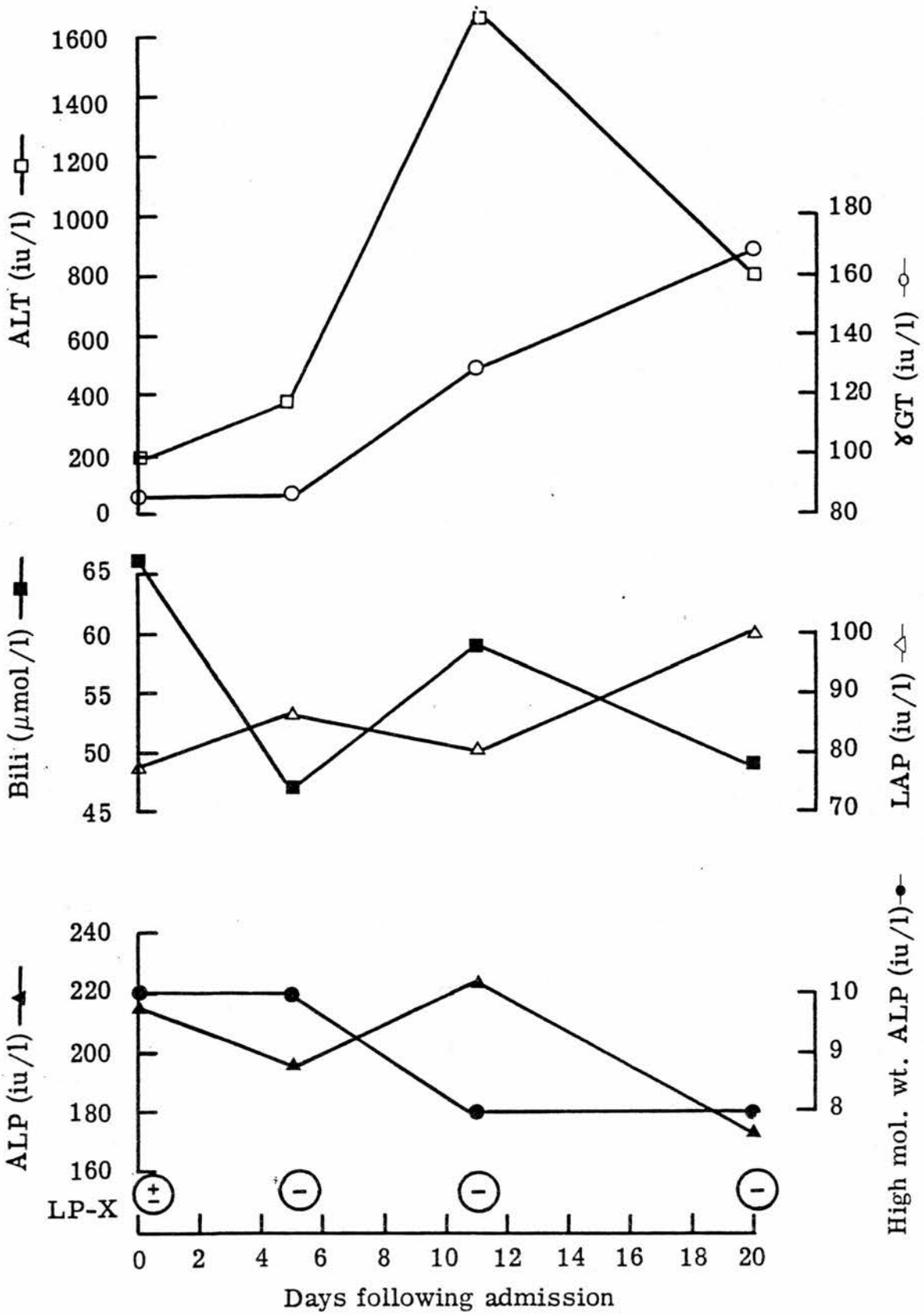


Figure 5.4. (continued).

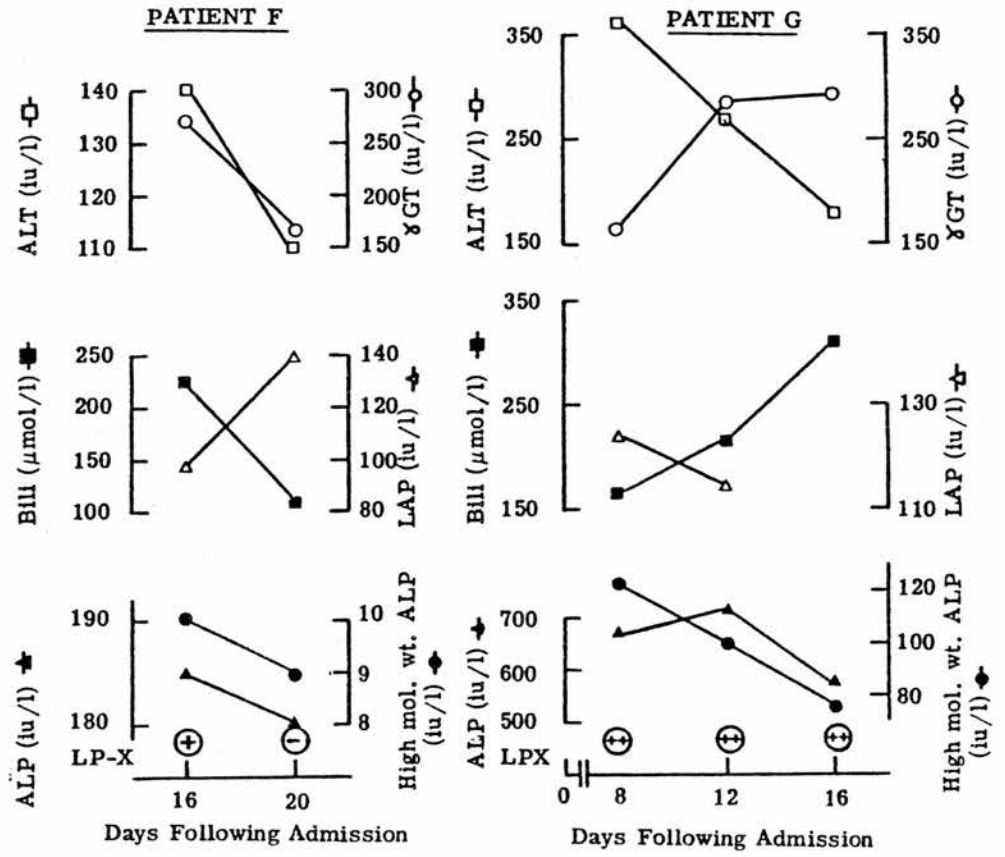
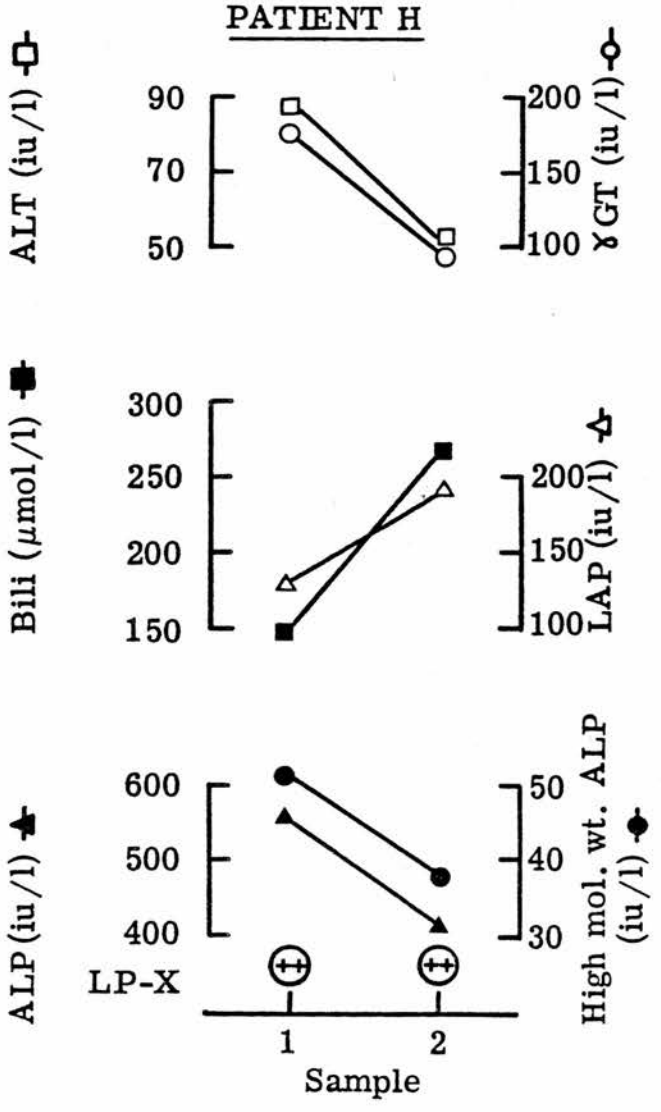


Figure 5.4. (continued).



enzyme activities, namely alanine aminotransferase, γGT and ALP varied in parallel and fell during the resolving phase of the illness. However, the magnitudes were very different: alanine aminotransferase reached 20 to 50 times, bilirubin 3 to 20 times, and γGT and ALP only 2 to 4 times the upper limit of their reference ranges. LAP often remained out of step with the other enzymes but it never rose above twice the upper limit of its reference range. The lack of correlation with the other enzymes may therefore have been largely due to chance fluctuations in serum, which owed more to physiological than to pathological mechanisms.

Patient D (Fig 5.4.), who also had chronic cardiac failure, had a secondary episode of cholestasis in which γGT, LAP and ALP activities in serum again rose in parallel but without an accompanying increase in bilirubin and alanine aminotransferase.

As far as high mol wt ALP was concerned, it generally rose to 4 to 6 times the levels seen in normal controls. It more closely paralleled the time course of the indices of cholestasis, namely total ALP, γGT and LAP, than the indices of cell necrosis, namely bilirubin and alanine aminotransferase, but did on occasion vary independently. General support for these observations came from sequential measurements in two patients with much higher levels of

high mol wt ALP in liver disease of an obstructive type, namely carcinoma of the head of the pancreas (patient G) and cholangiocarcinoma (patient H) (Fig 5.4.).

5.9. CONCLUSIONS

1) High mol wt ALP proved to be a sensitive and specific test for liver disease. It was higher a) in liver disease than in bone disease and normal controls, b) in obstructive lesions than in non-obstructive lesions and c) in patients with obstruction due to carcinoma in the liver or its vicinity than in non-neoplastic forms of obstruction. It was the best single test for diagnosing liver metastases and may therefore prove to be useful in the early detection of liver secondaries from a known primary carcinoma.
2) In the computer-assisted diagnosis of liver disease, high mol wt ALP appeared in many of the discriminant functions with a frequency second only to alanine aminotransferase. In particular, it appeared in most of the discriminant functions which distinguished metastatic liver disease from other liver diseases.
3) High mol wt ALP was released into the circulation in those pathological conditions characterised by intra- or extrahepatic cholestasis. Both single measurements in a number of patients and serial measurements in

individual patients showed that it correlated more closely with those variables generally regarded as indicating cholestasis than with those principally associated with cell necrosis. However, the correlation was not close and it seems likely that the pathophysiological factors governing the release of high mol wt ALP into the circulation are not identical to those governing the release of total ALP, γGT and LAP which are either partly or principally composed of low mol wt forms.

CHAPTER 6.

AN ELECTROPHORETICALLY SLOW-MOVING VARIANT OF ALKALINE PHOSPHATASE

6.1. INTRODUCTION

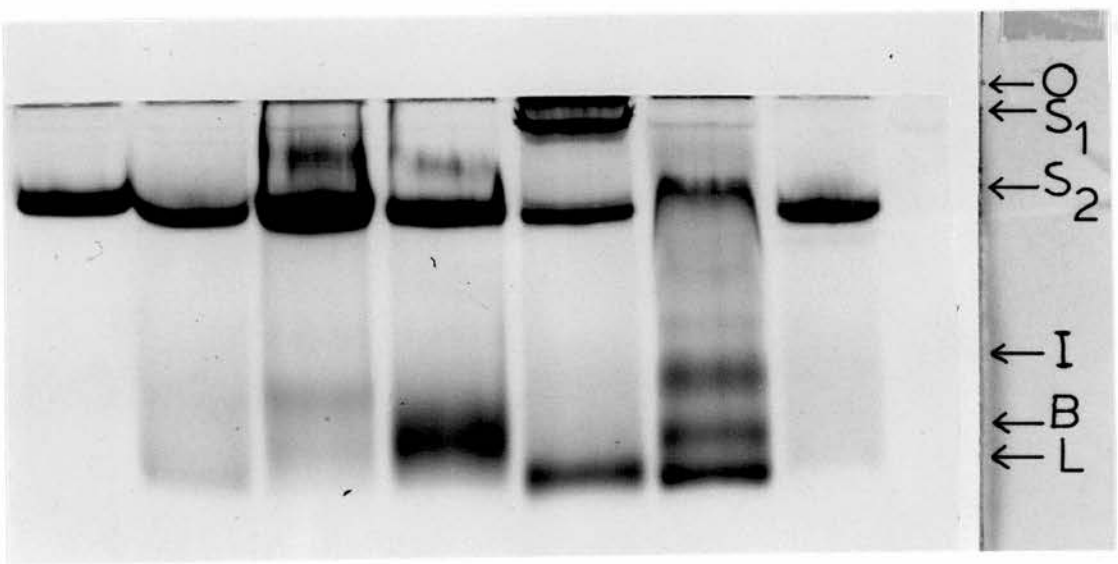
In addition to the high mol wt ALP which forms the principal object of study in this thesis, a second high mol wt form of ALP (designated slow band ALP to avoid confusion) was observed in the sera of 22 out of approximately 4500 patients whose serum ALP patterns were studied over a period of 52 months. Slow band ALP is therefore much rarer than high mol wt ALP. It migrated with about one third of the mobility of the liver isoenzyme during electrophoresis in 7% polyacrylamide gel, and had a mol wt intermediate between that of the liver isoenzyme and that of high mol wt ALP (see section 6.3.). The large size of slow band ALP might arise from aggregation of ALP molecules or association of ALP with a) protein b) lipid c) carbohydrate or d) a combination of these moieties. Its nature was therefore investigated with a view to determining whether it resembled the other high mol wt ALP, in any way. The role of slow band ALP in diagnosis was also assessed.

Using starch gel electrophoresis, an atypical slowly migrating zone of ALP activity (slow band) was first

reported by Streifler et al (1972) in a patient with ulcerative colitis. This was followed by a paper by Q◡irbi and Moss (1975) which described the occurrence of a similar slow band on polyacrylamide gel electrophoresis in a further 3 out of a total of 54 patients with ulcerative colitis or Crohn's disease. In each case the slow band formed between 50% and 100% of the total ALP activity. Isolated reports of similar slow bands have also appeared (Nagamine and Ohkuma, 1975; Lee, 1976). Dingjan et al (1975) found a slow band in 7 out of 2500 sera submitted for routine ALP isoenzyme analysis.

The present study investigates a strongly-staining slow band of ALP activity which moves with approximately one-third the mobility of the liver isoenzyme during electrophoresis in 7% polyacrylamide gel and forms 50% to 100% of the total ALP activity. Besides the 22 patients with this prominent slow band in their serum, many other sera had faint irregular bands with comparable mobility (e.g. patient M.G. in Fig 6.1.) but these looked qualitatively different from the prominent slow band under consideration. To avoid confusion, these commoner, irregular, faint slow bands will be termed 'irregular bands'. They were frequently multiple in number and usually occurred in sera containing the intestinal

Figure 6.1. ALP electrophoresis patterns on polyacrylamide gel. Sera from 6 of the patients studied are shown. The serum belonging to patient M.G., containing the irregular band, is included for comparison. Bands: O, origin; S_1, an anomalous slow band seen only in the serum of patient 13; S_2, the slow band under investigation; I, intestinal isoenzyme; B, bone isoenzyme; L, liver isoenzyme.



18 14 22 11 13 M.G. 1 +

PATIENTS

isoenzyme. The two types of slowly-migrating ALP were compared in all the investigations carried out. One serum only (patient 13, Fig 6.1.) contained a second prominent slow-moving band near the origin. This, because of its rarity, was not intensively investigated.

Previous investigations have not demonstrated whether the slow band is a single entity possessing identical properties in the diverse disorders in which it appears. In this study, sera from 8 patients with the slow band were studied in depth to determine its likely nature and origin.

6.2. CLINICAL SIGNIFICANCE

The age, sex and principal diagnosis of each of the patients in whom the slow band occurred are given in Table 6.1. The table also shows the total ALP activity of the sera. No particular association with age or sex is apparent although the band does tend to occur in the older age group. In no case did the total ALP activity rise above 4 times the upper limit of the reference range and usually it was below 250 iu/l. It is not known whether any patients with total ALP activity below 100 iu/l have the slow band since electrophoresis was not performed on such sera. The slow band occurred most frequently in patients with intestinal or lung disorders but the hetero-

TABLE 6.1.

Age, sex and clinical diagnosis of patients studied

Patient	Age (years)	Sex	ALP activity (iu/l)	Principal Diagnosis
				Pulmonary disease
1*	73	F	115	Chronic asthma
2*	78	M	147	Chronic bronchitis
3*	74	M	169	Chest infection
4	60	F	210	Chronic bronchitis
5	56	M	112	Lung neoplasm
6	51	M	121	Chest infection
7	60	F	124	Bronchiectasis
8	66	M	223	Tuberculosis
9	59	F	221	Lung collapse
				Intestinal disease
10	58	M	151	Coeliac disease
11*	57	F	231	Coeliac disease
12	50	M	185	Coeliac disease
13*	60	M	350	Ulcerative colitis
14*	33	F	149	Ulcerative colitis
15	65	M	122	Duodenal ulcer
16	45	M	112	Diarrhoea (unknown cause)
17		M	169	Diverticular disease
				Miscellaneous
18*	55	M	156	Thrombophebitis
19	63	F	114	Vasculitis
20	52	F	191	Psoriasis
21	75	F	160	Sub-acute combined degeneration of the spinal cord
22*	83	F	299	Breast adenocarcinoma
				Irregular band
M.G *	68	F	133	Coeliac disease

* patients studied in depth.

geneity of these makes it difficult to postulate a single underlying mechanism or to attach any clinical significance to its presence in serum. No association with any drug or group of drugs (e.g. steroids) was found. Serum protein and immunoglobulin concentrations were usually normal and protein electrophoresis showed no characteristic abnormalities.

Because similar slow bands have been previously reported in some cases of ulcerative colitis (Streifler et al, 1972; Qirbi and Moss, 1975) and because 2 patients in this series had ulcerative colitis, a further 9 sera from patients with established ulcerative colitis in various stages of activity were examined: none possessed the slow band. Three patients who were followed up one month later still had the slow band unchanged in their serum but no long-term follow-up studies were carried out.

6.3. MOLECULAR WEIGHT

A serum containing the slow band complex (patient 11) was applied to a Sepharose 6B column which had been calibrated with proteins of known mol wt. Fig 6.2. shows the position at which the slow band complex eluted relative to the liver isoenzyme. The mol wt was estimated to be 550 000. Polyacrylamide gel gradient electrophoresis (Fig 6.3.) yielded an estimate of 540 000 for the mol wt of the slow band complex (patient 1), a result in close

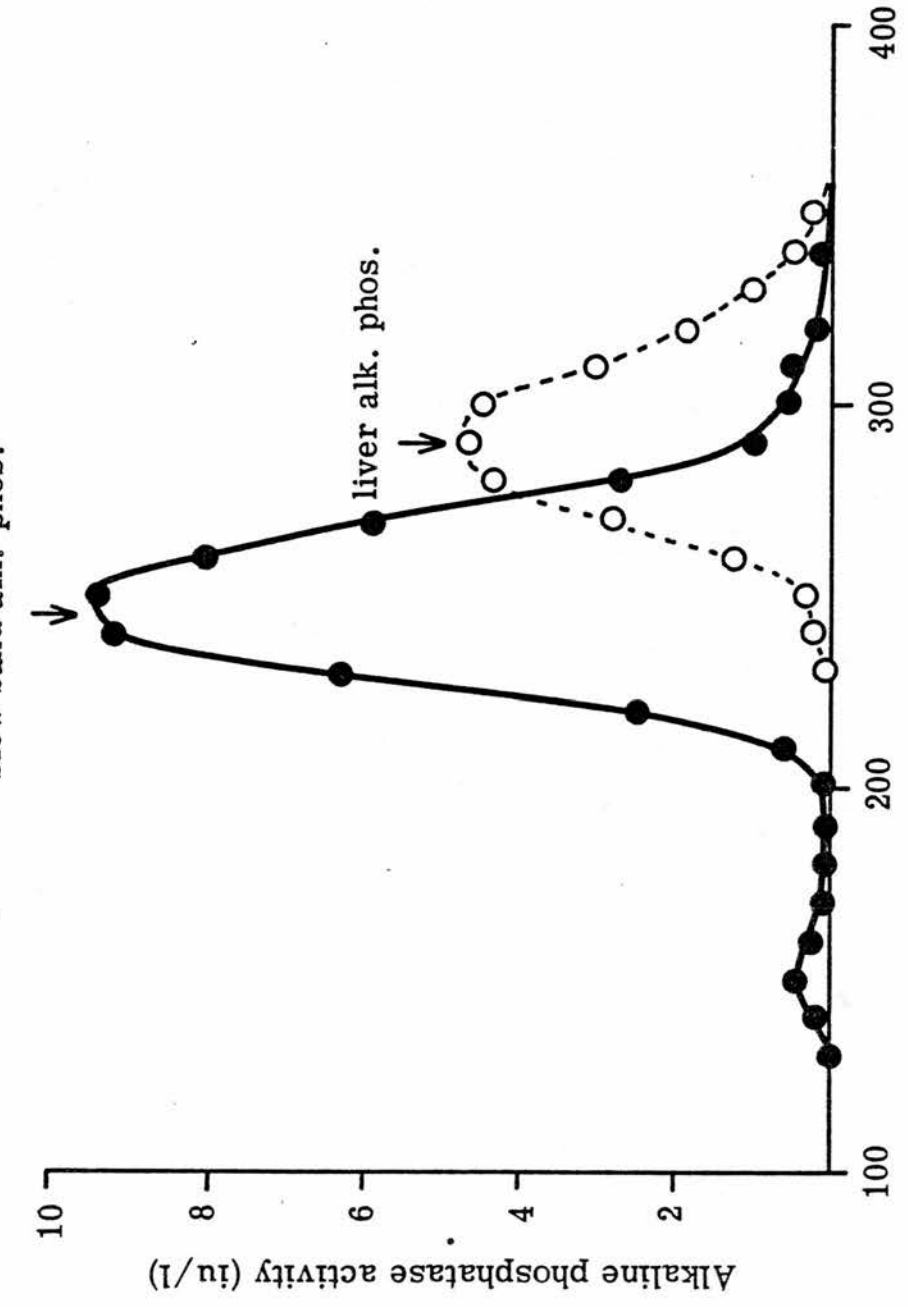


Figure 6.2. Elution patterns obtained by Sepharose 6B chromatography on a 95 x 2.5 cm column, following application of sera containing slow band ALP (patient 11) and liver ALP respectively.

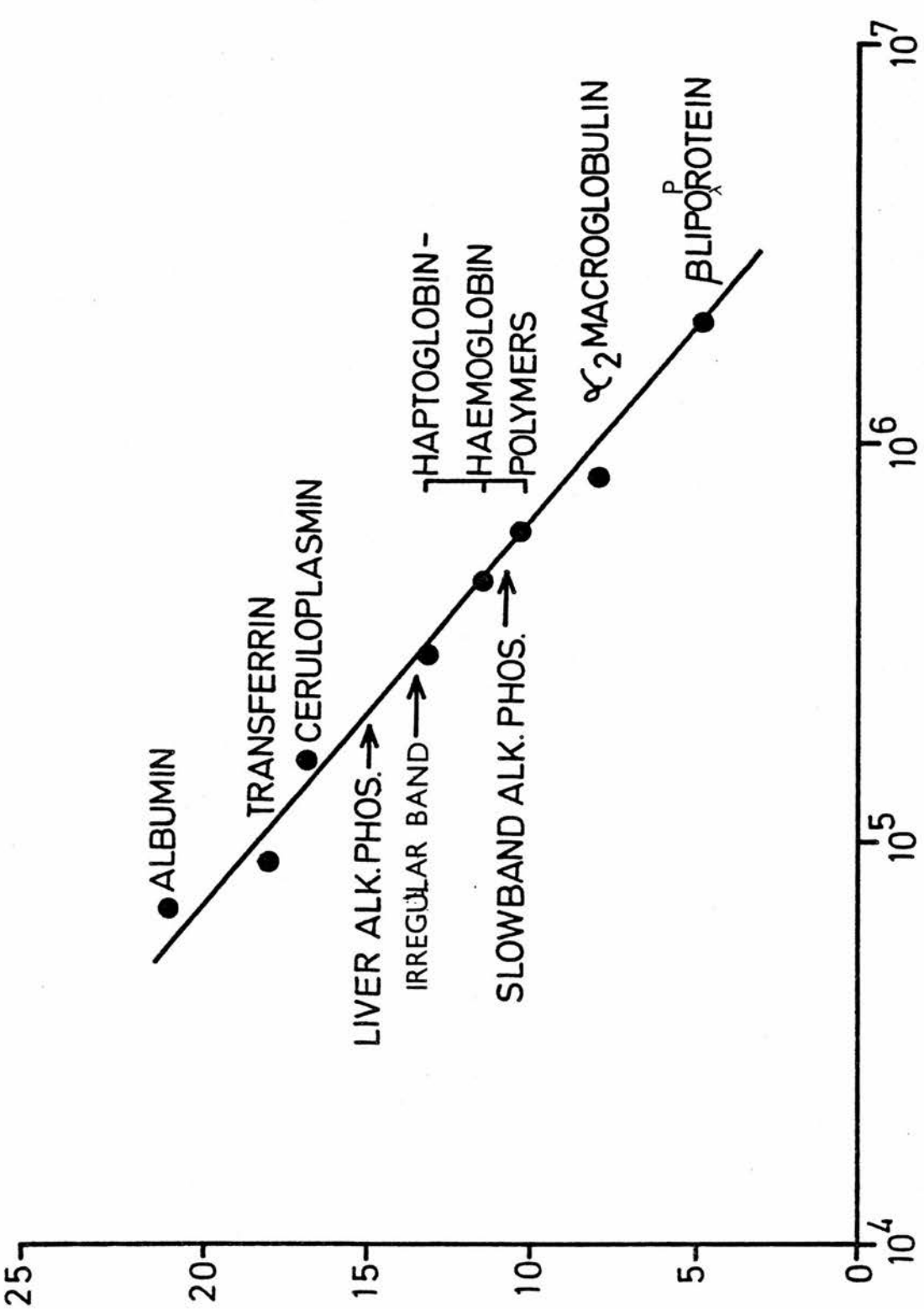


Figure 6.3. Mol wt calibration, using serum proteins, of a 4 to 26% polyacrylamide gradient gel. The gel concentrations to which the liver, slow band and irregular band ALPs migrated after electrophoresis to equilibrium are marked by arrows.

agreement with that from gel chromatography. The irregular band from patient M.G., also investigated for comparative purposes, was estimated by gradient gel electrophoresis to have a mol wt of 300 000.

6.4. CHARGE AND EFFECT OF INCUBATION WITH NEURAMINIDASE

Cellulose acetate, unlike polyacrylamide gel, exerts little or no retarding effect due to molecular size and thus afforded a better electrophoretic medium for assessing the charge of the slow band complex relative to the liver isoenzyme. In this medium, the slow band complex migrated very closely behind the liver isoenzyme. The charges of the two types of ALP must therefore have been similar.

Incubation with neuraminidase according to the method outlined in section 4.3 resulted in retardation of both liver and slow band ALP to approximately the same extent during electrophoresis in cellulose acetate (Table 6.2.) Very little retardation of the slow band occurred in 7% polyacrylamide gel since its mobility was already low in this medium owing to the molecular sieving effect. The effect of neuraminidase on the slow band was therefore masked. The intestinal isoenzyme, included for control purposes, was not retarded by incubation with neuraminidase. The mobility of the irregular band (patient M.G.) was little altered by treatment with neuraminidase.

TABLE 6.2.

Retardation of ALP bands by neuraminidase on two electrophoretic support media

	% Retardation of neuraminidase-treated isoenzyme	
Type of ALP	7% polyacrylamide gel	In cellulose acetate
Liver	44	20
Intestinal	0	0
Slow band	25	21
Irregular band	-12	-

If anything, it migrated slightly faster in the neuraminidase-treated serum.

The content of sialic acid residues therefore seems to be similar in the slow band complex and in the liver isoenzyme.

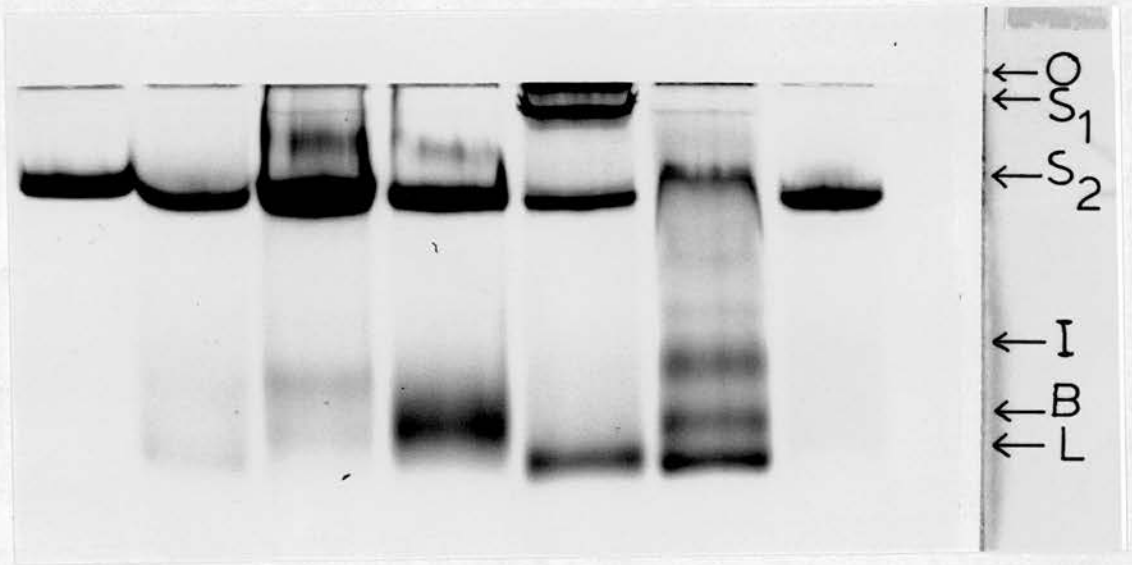
6.5. CHEMICAL INHIBITION

9 sera in which the slow band predominated, one serum containing the irregular band and 2 control sera in which the liver and intestinal isoenzymes respectively predominated were studied. ALP activities were measured in the presence and absence of 5 mmol/l L-homoarginine, L-phenylalanine and L-leucine. The sera were also subjected to electrophoresis in 7% polyacrylamide gel and stained for ALP activity in the presence of 5 mmol/l inhibitor. The resulting bands were compared with those of a control gel stained in the absence of inhibitor.

The qualitative effects of the inhibitors on the predominant electrophoresis band (Fig 6.4., Table 6.3.) showed that all the slow bands resembled the liver/bone rather than the intestinal isoenzyme. They were strongly inhibited by L-homoarginine, partially by L-leucine but only slightly by L-phenylalanine. This was in contrast to the intestinal isoenzyme and the irregular band (patient M.G.) which were both inhibited by L-phenylalanine but

Figure 6.4. ALP electrophoresis patterns in polyacrylamide gel stained in the presence of various inhibitors. Sera from 6 of the patients studied are shown. Serum from patient M.G., containing the irregular band, is included for comparison. Band notation as in Fig 6.1.

(a) Control gel: no inhibitor



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(b) 5 mmol/l L-homoarginine

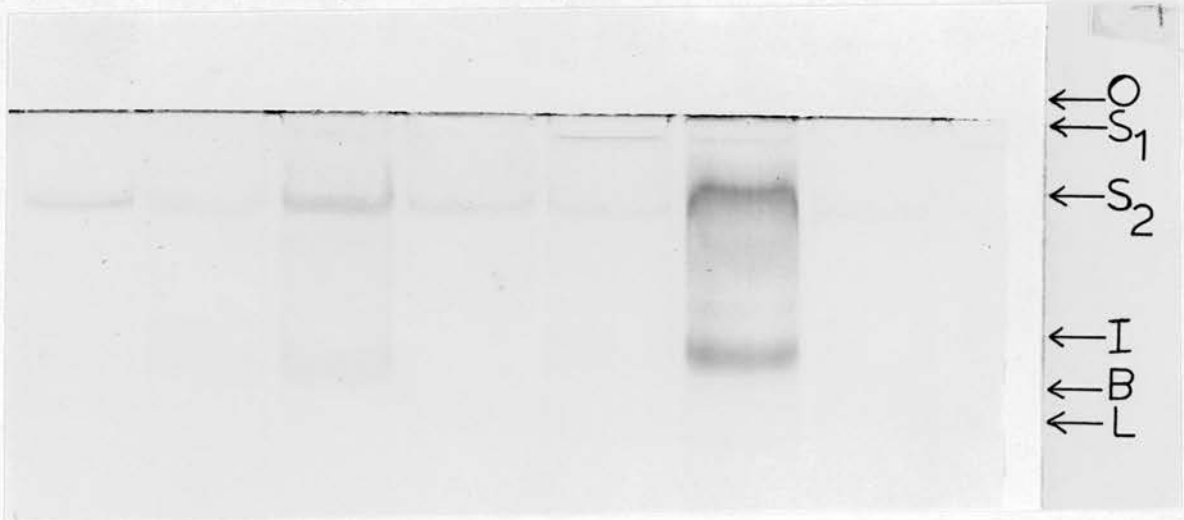

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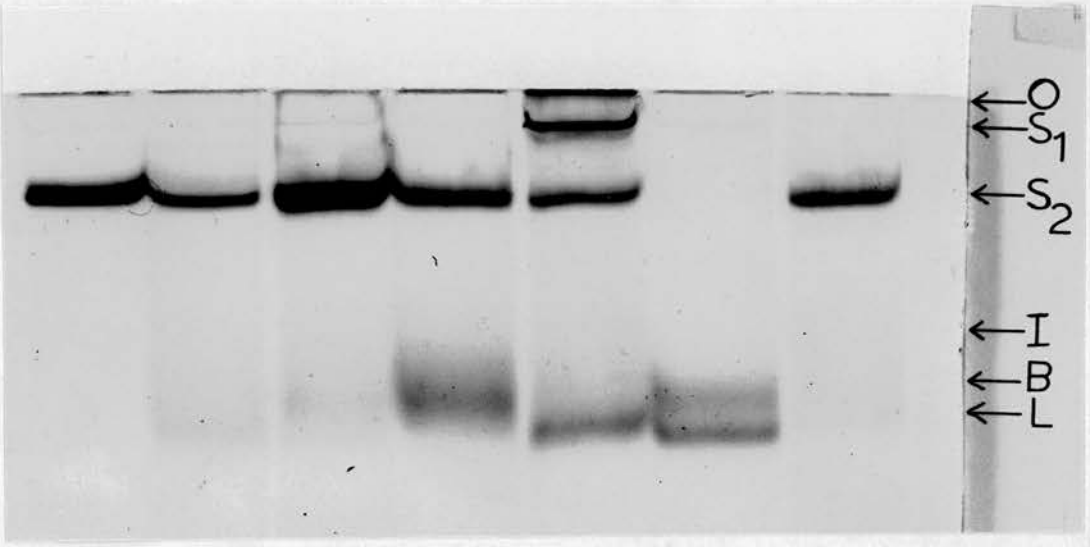
Figure 6.4. continued

(c) 5 mmol/l L-phenylalanine



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(d) 5 mmol/l L-leucine

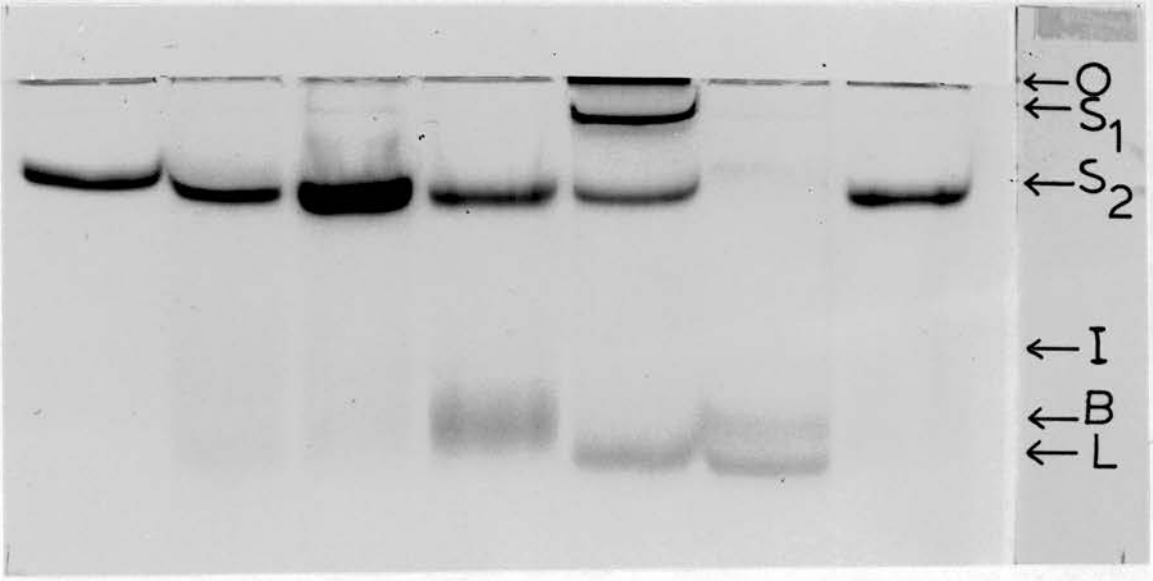

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TABLE 6.3.

Qualitative effects of inhibitors on slow band and irregular band ALP compared with liver and intestinal isoenzymes

Predominant isoenzyme in serum	Qualitative effect on predominant electrophoresis band		
	L-Homoarg.	L-Phe.	L-Leu.
Intestinal	Resistant	Sensitive	Partially sensitive
Irregular band (patient M.G.)	Resistant	Sensitive	Partially sensitive
Liver	Sensitive	Resistant	Partially sensitive
Slow band			
patients 1	Sensitive	Resistant	Partially sensitive
2	Sensitive	Resistant	Partially sensitive
3	Sensitive	Resistant	Partially sensitive
4	Sensitive	Resistant	Partially sensitive
11	Sensitive	Resistant	Partially sensitive
13	Sensitive	Resistant	Partially sensitive
14	Sensitive	Resistant	Partially sensitive
18	Sensitive	Resistant	Partially sensitive
22	Sensitive	Resistant	Partially sensitive
"Slower" band patient 13	Sensitive	Resistant	Resistant

TABLE 6.4.

Inhibition of slow band and irregular band ALP compared with liver and intestinal isoenzymes

Predominant isoenzyme in serum	% inhibition of total activity			
	L-Homoarg	L-Phe	L-Leu	Urea
Intestinal	65	24	35	-
Irregular band (patient M.G.)	64	20	32	56
Liver	73	15	31	47
Bone	-	-	-	64
Slow band				
patient 1	71	20	41	48
2	-	-	-	51
3	-	-	-	50
11	70	10	29	59
13	71	14	8	-
14	70	14	30	46
18	71	17	33	46
22	72	14	30	66

were largely resistant to inhibition by L-homoarginine. The quantitative studies (Table 6.4.) showed a similar pattern in most cases but the results were probably partly obscured by the presence of other isoenzymes in the sera. The extra slow band near the origin in patient 13 (S_1 in Fig 6.4.) also behaved like the liver isoenzyme except that it was resistant to inhibition by L-leucine. The effect of this additional slow band probably explains the reduced quantitative sensitivity to L-leucine shown by serum 13.

In summary, all the slow bands resembled the liver/bone isoenzyme whereas the irregular band (patient M.G.) resembled the intestinal isoenzyme in their responses to these inhibitors.

The effect of 2 mol/l urea on the ALP activities of 7 sera containing the slow band is also shown in Table 6.4. 5 of the slow band sera were inhibited by urea to about the same extent as the liver isoenzyme whereas the other two sera (patients 11 and 22) behaved more like the bone isoenzyme. Both had bone isoenzyme activity in their serum (Fig 6.4.(a)) in addition to the slow band and this may have accounted for the greater inhibition by urea shown by these sera. In other words, the evidence is insufficient to conclude whether or not the slow band may have been

derived from the bone rather than the liver isoenzyme in these two sera. Serum from patient M.G. showed an intermediate degree of inhibition, probably accounted for by the presence of appreciable amounts of liver, bone and intestinal isoenzymes as well as the irregular band. The results of urea inhibition of both the slow and the irregular band must therefore be regarded as equivocal.

6.6. INACTIVATION BY HEAT

Heat inactivation curves were determined on whole sera by the method of Whitby and Moss (1975). To determine the qualitative effect of heat on the individual isoenzymes, the sera were heated at 56°C (± 0.2°C) for 10 minutes (Johnson et al., 1972) and then cooled rapidly. The ALP isoenzymes in the heated sera and in the unheated controls were separated on 7% polyacrylamide gel. A similar experiment was carried out to determine the effect of heating at 65°C for 5 minutes.

The serum from patient 18 was selected for study because its ALP was virtually entirely in the slow band form (Fig 6.1.). On heating at 56°C, a biphasic exponential decay curve was obtained (Fig 6.5. Table 6.5.). The first component of this curve comprised one-third of the total activity and was slightly more heat labile than the bone isoenzyme; the second component formed two-thirds of

Figure 6.5. The effect of heating at 56°C on ALP activity in whole serum.

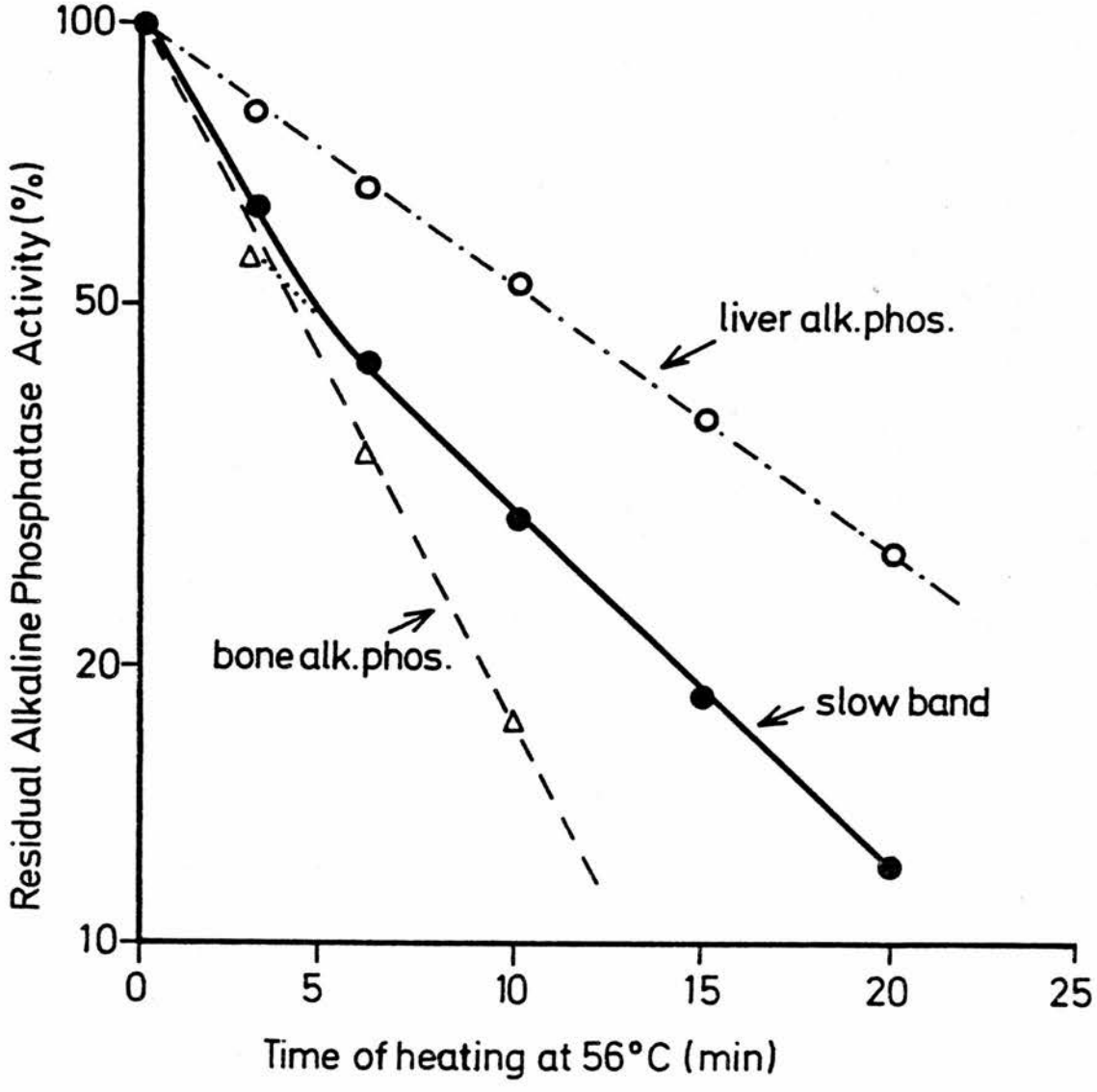


TABLE 6.5.

The half-lives at 56°C of the liver and bone isoenzymes and of slow band ALP in whole serum and the percentage of each component in the biphasic exponential decay curve (patient 18)

ALP isoenzyme	Percentage of component		Half-life of component (min)	
	Heat-labile	Heat-stable	Heat-labile	Heat-stable
Liver*	-	100		10.4
Bone and Liver	86.4	13.5	3.1	15.2
Slow band	33.7	66.3	2.0	8.1

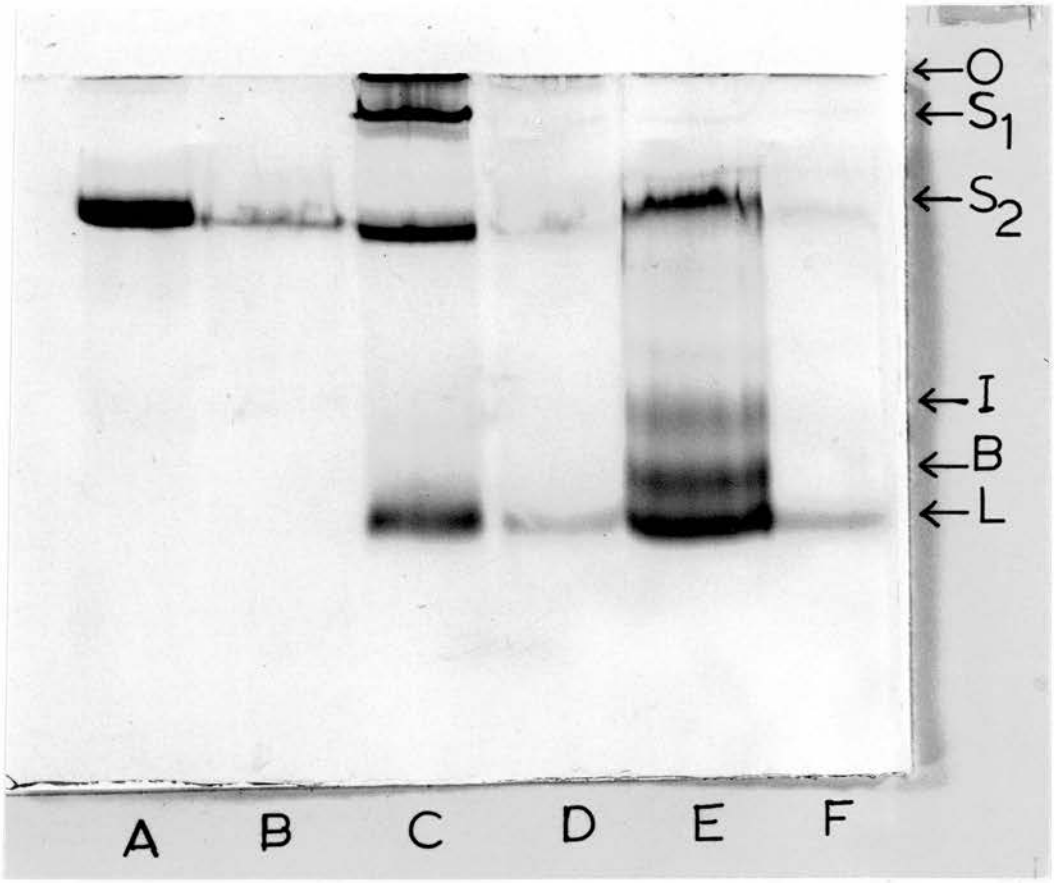
* Curve monoexponential; half-life estimated graphically.

the total activity and was more heat-labile than the liver isoenzyme. It is possible, therefore, that the slow band complex in this serum contained both liver and bone isoenzymes which were both rendered slightly more heat-labile by their attachment to the complex. Serum from patient M.G. (not shown) had an overall ALP half-life of 5.6 minutes but the half-life of the irregular band could not be separately estimated owing to the mixture of isoenzymes in this serum. Qualitative electrophoretic studies on the effect of heating at 56°C for 10 minutes (Fig 6.6.) confirmed the heat lability of the slow band complex. The 'slower' band in patient 13 (S_1 in Fig 6.6.) seemed to be even more heat-labile than the slow band and disappeared completely after heating at 56°C for 10 minutes. Heating at 65°C for 5 minutes destroyed all the slow band activity as well as the irregular band and the liver, bone and intestinal isoenzymes.

6.7. ASSOCIATION WITH IgG

Immunoelectrophoresis against antisera to human IgG, IgA, IgM and K and λ light chains (Scheidegger, 1955) was performed in duplicate on 9 sera containing the slow band, on serum from patient M.G. and on sera containing the liver, bone and intestinal isoenzymes. After washing the plates in saline for 2 days, they were stained for a) protein and

Figure 6.6. The effect of heating at 56°C for 10 minutes on ALP electrophoresis patterns in polyacrylamide gel. A, patient 18 control serum; B, patient 18 serum after heating; C, patient 13 control serum; D, patient 13 serum after heating; E, patient M.G. control serum; F, patient M.G. serum after heating. Band notation as in Fig 6.1.



b) ALP.

ALP activity was associated with the IgG precipitin arc in all sera containing the slow band (Fig 6.7., Table 6.6.). No ALP activity was present in the IgA or IgM precipitin arcs of these sera, nor was there any enzyme activity in any of the precipitin arcs formed by the control sera or serum from patient M.G. with the irregular band. In most of the sera containing the slow band, it was possible to demonstrate ALP activity in the arcs formed against either the K or the λ light chain antisera - but never both. The equivocal results sometimes obtained were possibly due to poorly reacting antisera.

In order to confirm an association between the slow band and IgG, an attempt was made to demonstrate directly the presence of IgG in the slow band. After polyacrylamide gel electrophoresis of a serum containing the slow band, protein was eluted electrophoretically from the section of gel surrounding the slow band. Other sections of gel corresponding to the region of the liver isoenzyme and the intermediate region were also eluted as controls. An attempt was made to demonstrate the presence of IgG in the eluates but IgG was undetectable by nephelometry and by Laurell rocket electroimmunoassay (Laurell, 1966). Assuming a specific activity for ALP of 1450 iu/mg

Figure 6.7. Immunoelectrophoresis pattern stained for ALP activity, using serum from patient 13 (W.B.) and a control serum.

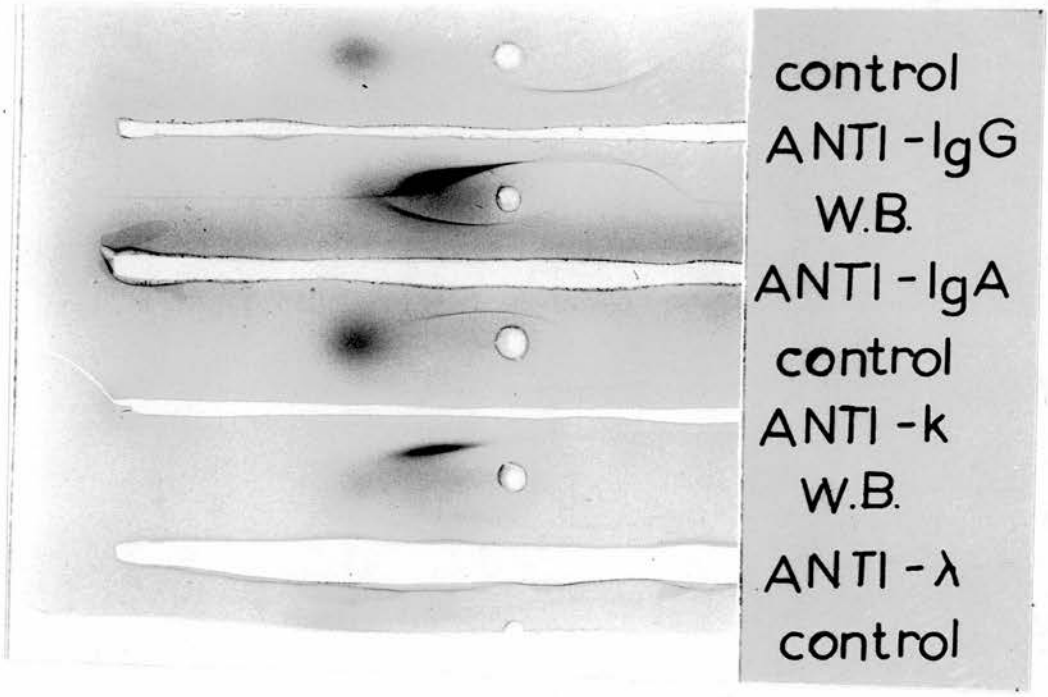


TABLE 6.6.

Immunoelectrophoresis of sera containing slow bands compared with control sera

Predominant isoenzyme in serum	Precipitin arcs with ALP activity	
	Immunoglobulin class	Light chain type
Liver	None	None
Bone	None	None
Intestinal	None	None
Irregular band (patient M.G.)	None	None
Slow band		
Patient 1	IgG	λ
2	IgG	κ
3	IgG	λ
11	IgG	? λ
13	IgG	κ
14	IgG	? λ
15	IgG	κ
18	IgG	λ
22	IgG	κ

(Latner and Hodson, 1976) and that one molecule of ALP combines with a maximum of two molecules of IgG, the expected concentration of the ALP-specific IgG can be calculated as follows. A serum with a slow band activity of 150 iu/l contains 0.1 mg/l, or about 0.5 nmol/l ALP protein which will combine with 1 nmol/l or about 0.15 mg/l IgG. This is $1:10^5$ of the normal serum concentration and thus below the limits of detection of the methods of measuring IgG which were used.

An indirect experiment involving precipitation by antiserum was therefore carried out. The following combinations of ALP-containing human sera and anti-immunoglobulin antisera were incubated at $4^{o}C$ for 24 hours in the ratio 1 volume serum:5 volumes antiserum:

a) Liver isoenzyme serum (control) + IgA antiserum

b) Liver isoenzyme serum (control) + IgG antiserum

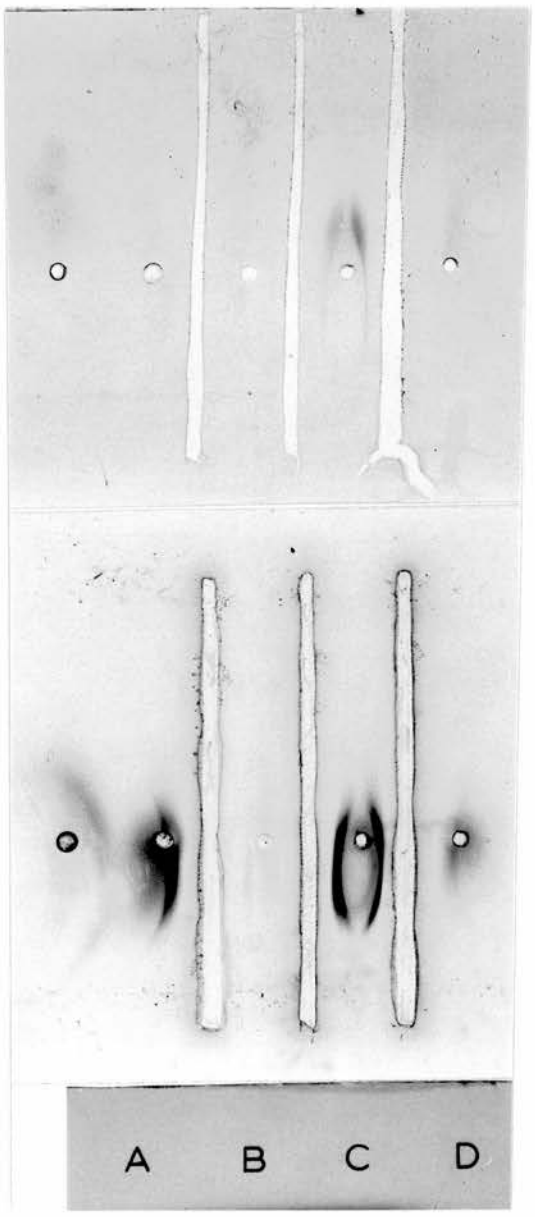
c) Slow band serum (patient 3) + IgA antiserum

d) Slow band serum (patient 3) + IgG antiserum

The mixtures were then centrifuged to remove any precipitated antigen-antibody complexes. Immunoelectrophoresis of the supernatants against IgG antiserum followed by staining for both ALP activity and protein showed, for each of the above incubation mixtures (Fig 6.8.):

a) IgG present; no ALP arc

Figure 6.8. Immunoelectrophoresis using IgG antiserum. The upper plate is stained for ALP activity and the lower plate is stained for protein. A, supernatant from incubation (a); B, supernatant from incubation (b); C, supernatant from incubation (c); D, supernatant from incubation (d). See text for details.



b) No IgG present; no ALP arc

c) IgG present associated with ALP activity

d) No IgG present; no ALP arc.

The anti-IgG antiserum had therefore been successful in precipitating all detectable IgG in the sera.

Electrophoresis of the supernatants in 7% polyacrylamide gel showed that the slow band had disappeared from the serum incubated with anti-IgG (d) but not from the serum incubated with anti-IgA (c). The liver isoenzyme was unaffected by either anti-IgG (b) or anti-IgA (a) (Fig 6.9.). These experiments demonstrate conclusively that the slow band consists of a complex between ALP and IgG.

The following experiment was performed to determine whether the sera containing the slow band were capable of binding ALP isoenzymes added from other sources, in other words, whether there was spare binding capacity. One volume of a slow band serum (patient 2) was incubated for 24 hours at $4^{\circ}C$ with one volume of each of the following sera respectively:

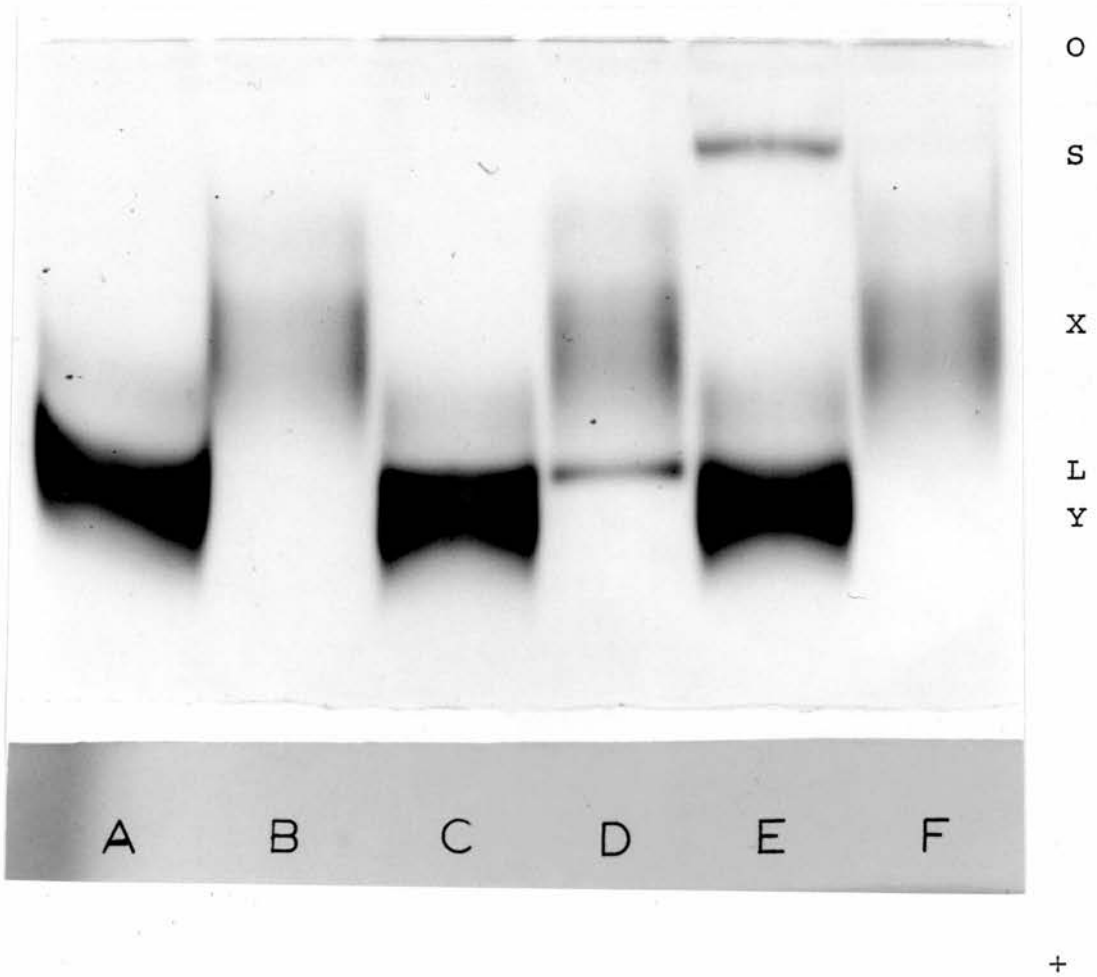
a) liver ALP serum

b) bone ALP serum

c) intestinal and liver ALP serum.

As controls, each serum was incubated separately with the

Figure 6.9. ALP electrophoresis patterns in polyacrylamide gel showing the effect of removal of IgG from a serum containing the slow band. A, IgA antiserum control; B, IgG antiserum control; C, supernatant from incubation (a); D, supernatant from incubation (b); E, supernatant from incubation (c); F, supernatant from incubation (d); Bands: S, slow band; X, IgG antiserum ALP; L, liver isoenzyme; Y, IgA antiserum ALP. See text for details.



appropriate volume of saline alone. Subsequent electrophoresis on polyacrylamide gel (Fig 6.10.) showed that in incubations (a), (b) and (c) the slow band had been augmented and the liver and bone isoenzyme bands respectively had been diminished compared with the controls. However, in incubation (c), only the liver isoenzyme band was diminished compared with the control: no change was observed in the intestinal isoenzyme band. This suggests that there was indeed spare capacity of IgG in the slow band sera which appeared to bind liver or bone but not intestinal ALP to form the slow band complex.

Attempts to generate the slow band complex by incubation of sera containing various ALP isoenzymes, and also partially purified preparations of the isoenzymes with purified polyclonal human γ-globulin [Sigma Cohn Fraction II] were unsuccessful.

6.8. EFFECT OF DISSOCIATING AGENTS

Sera containing the slow band complex were treated with various dissociating agents as shown in Table 6.7. Electrophoresis was then performed in polyacrylamide gel containing either dissociating agent or no dissociating agent as shown in the table. Staining for ALP activity was invariably done in the absence of dissociating agent (and required prior soaking of the gel after electrophoresis

Figure 6.10. ALP electrophoresis patterns in polyacrylamide gel showing binding of other isoenzymes in the slow band serum to form the slow band. A, slow band serum (patient 2) + liver isoenzyme serum; B, slow band serum + bone isoenzyme serum; C, slow band serum + intestinal and liver isoenzymes serum; D, slow band serum control; E, liver isoenzyme serum control; F, bone isoenzyme serum control; G, liver and intestinal isoenzymes serum control. Band notation as in Fig 6.1. See text for details.

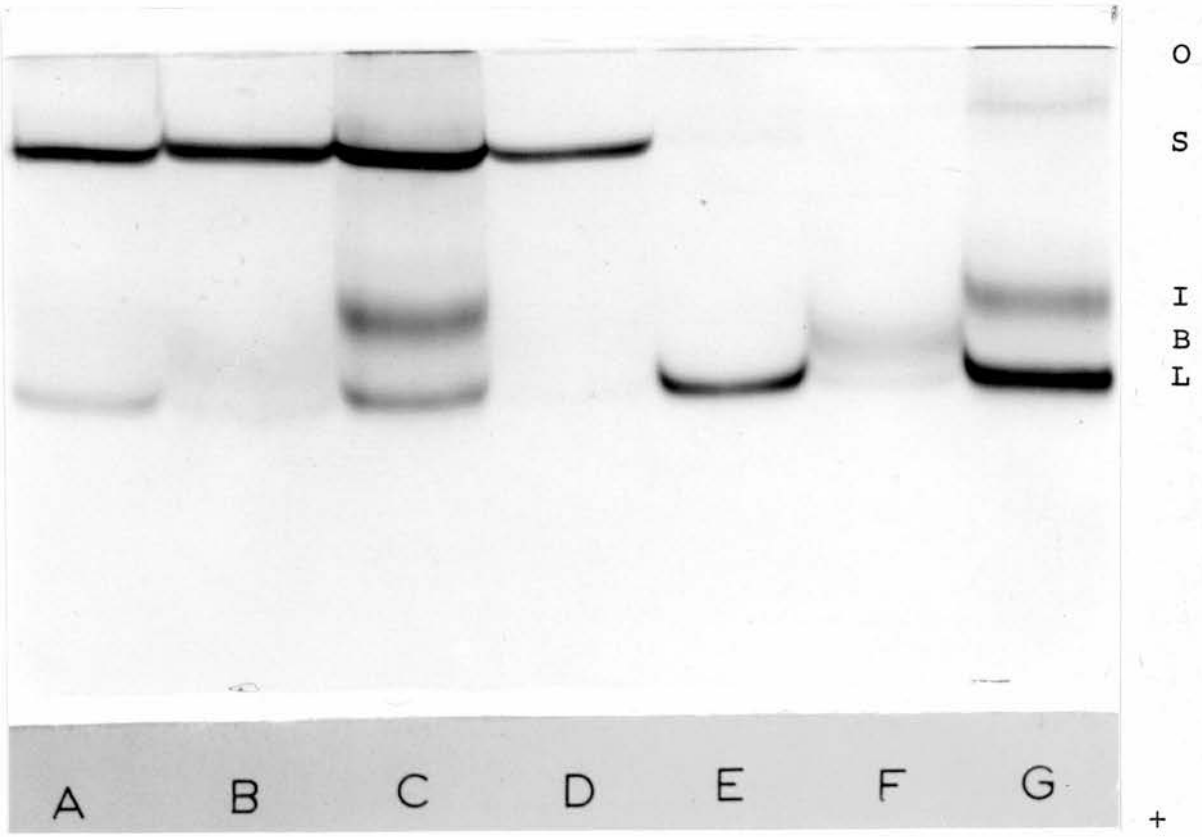


TABLE 6.7.

Effect of dissociating agents on the slow band complex

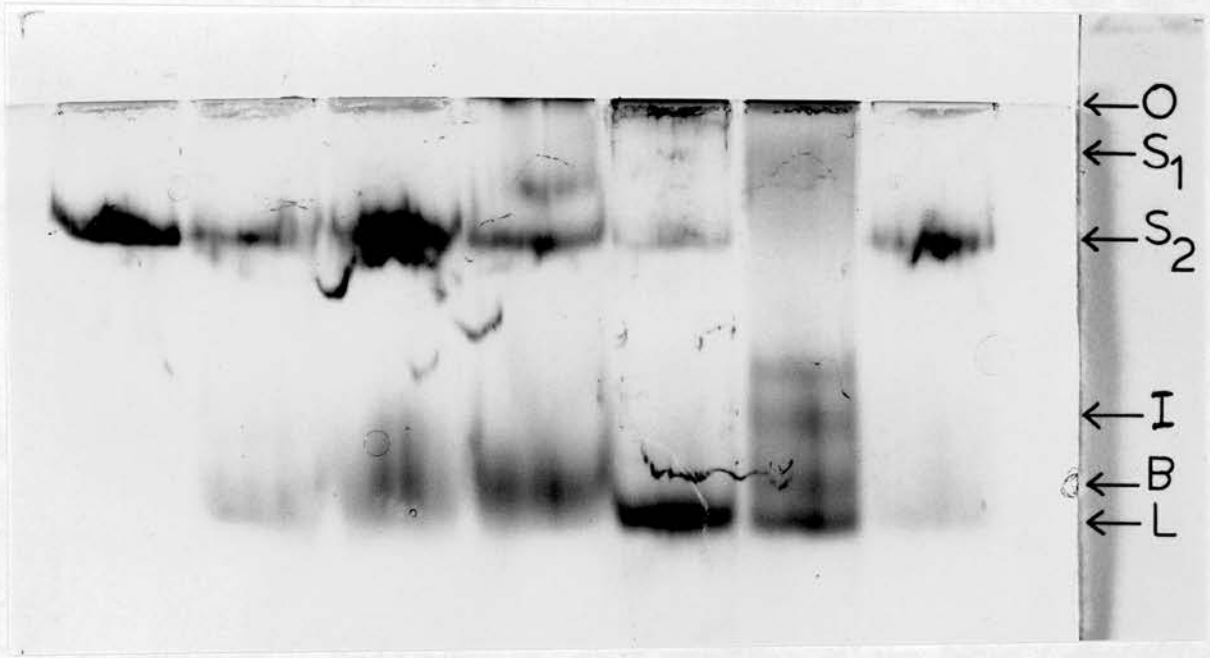
Dissociating agent	Incorporation of dissociating agent into polyacrylamide gel	Effect on slow band complex
1. 1% SDS:0.1% mercapto-ethanol	0.1% SDS	None
2. 1% Triton X-100	0.1% Triton X-100	None
3. di-isopropyl ether: butan-1-ol (60:40 v/v) 1 vol serum: 2 vols solvent	None	None
4. 8 mol/l urea	8 mol/l urea None	No activity observed No activity observed
5. 8 mol/l guanidine hydrochloride	8 mol/l guanidine hydrochloride None	No activity observed No activity observed
6. Acidification using 0.1 mol/l citrate buffer pH 3.5.	0.1 mol/l citrate buffer pH 3.5 None (alkaline buffer used)	No activity observed No activity observed

in several changes of buffer in the urea and guanidine hydrochloride experiments).

The detergents, sodium dodecyl sulphate (SDS) and Triton X-100, both of which interfere with hydrophobic interactions, had no effect on the integrity of the slow band complex. However, SDS did abolish the second 'slower' band in the serum of patient 13 (Fig 6.11. c.f. Fig 6.1.), possibly converting it to a faster moving form. Similarly, the irregular band in the serum from patient M.G. disappeared and a new band appeared migrating closely behind the intestinal isoenzyme.

The organic solvent butan-1-ol (combined with di-isopropyl ether to mitigate its denaturing effects, as described by Cham and Knowles, 1976) which extracts lipophilic substances had no effect on any of the ALP bands of activity. Urea and guanidine hydrochloride, which disrupt hydrogen bonding, both irreversibly inactivated ALP in this experiment. This was because ALP is irreversibly inactivated at 8 mol/l urea, whereas at 2 mol/l (used in section 6.5.) the inhibition is reversible and of a non-competitive type (Birkett et al, 1967). Irreversible inactivation also occurred with acidification which has been used to disrupt protein-immunoglobulin linkages (Singer and Campbell, 1955). Since its enzymic

Figure 6.11. ALP electrophoresis patterns in polyacrylamide gel containing 0.1% SDS. Sera from 6 patients possessing the slow band, and serum from patient M.G. were treated with 1% SDS before electrophoresis. Band notation as in Fig 6.1. The patterns (poor quality owing to the effect of the detergent) should be compared with those in Fig 6.1.



activity was the only means of detecting and locating the slow band complex, it was not possible to tell whether these last three dissociating agents were successful or not in splitting up the slow band complex.

6.9. DISCUSSION AND CONCLUSIONS

To avoid confusion with the high mol wt ALP considered elsewhere in this thesis, the individual findings relating to the slow band complex will be considered here. The two forms of high mol wt enzyme will then be briefly compared and contrasted in Chapter 7.

All the properties tested in a number of sera containing the slow band complex were similar. It seems that the slow band complex is therefore a single entity with definable properties in all patients in whose sera it occurs. As a comparison, serum from patient M.G., which contained the irregular band, was included in the study as being representative of the many sera in which faint, irregular, sometimes multiple, slowly-migrating bands are seen on routine electrophoresis. These sera generally also contain the intestinal isoenzyme.

6.9.1. Inhibition

Inhibition studies using L-homoarginine, L-phenylalanine and L-leucine demonstrated that the slow band complex resembled liver or bone ALP rather than the

intestinal isoenzyme. Similarly, Dingjan et al (1975) and Nagamine and Ohkuma (1975) found that the slow band complex was resistant to inhibition by L-phenylalanine. By contrast, the irregular band resembled the intestinal isoenzyme in its inhibition properties.

6.9.2. Charge and neuraminidase treatment

In cellulose acetate which exerts little or no retarding effect due to molecular size, the slow band complex runs in the bone isoenzyme position, very closely behind the liver isoenzyme, suggesting that the charges of all three forms of ALP are similar. It is the higher mol wt of the slow band complex which retards it in polyacrylamide gel. Using polyacrylamide gel electrophoresis, Qirbi and Moss (1975) and Dingjan et al (1975) found almost no change in mobility of the slow band following treatment with neuraminidase. By contrast, using agar gel electrophoresis Nagamine and Ohkuma (1975) demonstrated a reduction in mobility. The present study confirms both findings. It seems likely that the molecular sieving action of polyacrylamide gel masks a true alteration in charge which is apparent in cellulose acetate. The slow band complex therefore resembles the liver and bone isoenzymes in the number and accessibility of its sialic acid residues. The irregular band, on the other hand, altered

its mobility very little when treated with neuraminidase and resembles the intestinal isoenzyme in this respect.

6.9.3. Inactivation by heat and urea

In the properties so far discussed the liver and bone isoenzymes behave identically. Urea inactivation studies were undertaken in an attempt to discover whether the slow band resembled the liver or the bone isoenzyme, but the results were equivocal. However, heat inactivation studies suggested that the slow band complex might contain both liver and bone isoenzymes, both of which might be rendered slightly more heat labile by their presence in the complex.

6.9.4. Association with IgG

ALP activity was associated with the precipitin arc against IgG antiserum in all 9 sera possessing the slow band in which immunoelectrophoresis was performed, thereby confirming and extending the observation of Nagamine and Ohkuma (1975). The light chain type varied from serum to serum, but in any one serum only one type of light chain was associated with ALP activity, suggesting that the IgG might be monoclonal. No IgG-associated ALP activity was detectable in control sera nor in serum from patient M.G. containing the irregular band.

Too little ALP-associated IgG was present in the

slow band sera for it to be measured directly. Instead, removal of IgG from a patient's serum by immuno-precipitation was accompanied by a concomitant removal of the slow band. Failure to generate the slow band by incubation of the liver, bone and intestinal isoenzymes with human polyclonal IgG implied that the ALP-associated IgG may be a monoclonal immunoglobulin not widely present in the population. That it may be present in excess in the slow band sera is suggested by the experiment in which incubation of a slow band serum with various control sera resulted in an enhancement of the slow band and a diminution of the liver and bone isoenzyme bands, but not of the intestinal isoenzyme band. Since the liver and bone isoenzymes are similar in their antigenic properties and differ from the intestinal isoenzyme in this respect (Boyer, 1963), it is possible that this binding occurs as an antigen-antibody-type reaction in vivo. Alternatively, the binding could be a non-specific attachment to the Fc fragment of IgG via the carbohydrate moiety, such as occurs between complement and IgG (Latner, 1975). In either case, it appears that IgG will bind any liver and bone isoenzymes present in the serum of the patient.

Nagamine and Ohkuma (1975) found that the slow band in their patient eluted from a Sephadex G200 gel in a

position intermediate between that of the liver isoenzyme and high mol wt ALP, but they did not measure the mol wt. The present study found a mol wt of 540 000 for the slow band complex. This suggests that the complex may consist either of two IgG molecules (mol wt 160 000) associated with one liver or bone ALP molecule (mol wt 220 000), or of two ALP molecules associated with one IgG molecule, the mol wt of 600 000 for the latter complex probably being within the analytical error for the mol wt estimates. The latter possibility is more consistent with the divalent nature of immunoglobulins.

6.9.5. Effect of dissociating agents

Attempts to dissociate the slow band complex using various agents were uniformly unsuccessful, suggesting that strong bonds other than disulphide bonds were involved. Only the 'slower' band in patient 13 and the irregular band in patient M.G. may have been dissociated by SDS. This detergent, unlike Triton X-100, frequently disrupts hydrophobic linkages between adjacent polypeptide chains. Both these complexes may therefore have been aggregates of lower mol wt isoenzymes. In the case of patient M.G., the evidence suggests that the irregular band was probably an aggregate of the intestinal isoenzyme. Other similar bands in sera may also be intestinal

isoenzyme aggregates and the multiple bands frequently observed in the same sera may represent aggregates of increasing numbers of monomers.

6.9.6. Clinical implications

Streifler et al (1972) found a strongly-staining slow band in a patient with ulcerative colitis. This initial report was followed up by Qirbi and Moss (1975) who reported the presence of the band in 2 out of 22 cases of ulcerative colitis and 1 out of 32 cases of Crohn's disease but in none of 33 patients with other diseases of the intestinal tract. Of the 7 patients with the strong slow band described by Dingjan et al (1975), only one had ulcerative colitis and another had a duodenal ulcer. 2 further patients had chronic bronchitis, one with carcinoma of the bronchus in addition. The remaining 3 patients had a variety of conditions. The patients investigated by Nagamine and Ohkuma (1975) and Lee (1976) had skeletal abnormalities and congestive cardiac failure respectively with no evidence presented of intestinal or lung pathology. In the present study, 8 of the 22 patients had intestinal disorders and 9 had lung disorders. The remaining 5 patients had a variety of diseases with no apparent common feature. This is in agreement with previous reports. Only 2 patients had ulcerative colitis and in a further 9

cases of ulcerative colitis examined no strong slow bands could be detected. Since steroid administration affects the immune response and is a therapy frequently used in such conditions as ulcerative colitis and chronic asthma, the drugs taken by the patients in this series were checked. No correlation was found between the presence of the slow band and administration of steroids or any other drug.

6.9.7. Other enzyme-immunoglobulin complexes

ALP is not unique in forming a complex with an immunoglobulin. Lactate dehydrogenase has been shown in a series of papers to form complexes in vivo with IgA (Biewenga and Feltkamp, 1975) of variable light chain type (Biewenga and Van Loghem, 1978). Amylase may form complexes in vivo with either IgG or IgA to form macro-amylase (Levitt and Cooperband, 1968; Hansen et al, 1972; Harada et al, 1975; Kanno and Sudo, 1977; Kobayashi et al, 1978). The light chain type of these complexes is also variable. Kaczmarek and Rosenmund (1977) succeeded in generating a macroamylase complex in vitro by incubating purified amylase with polyclonal human γ globulin. There have also been isolated reports of an aspartate amino-transferase-IgG complex (Konttinen et al, 1978) and a creatine kinase BB isoenzyme-IgG complex (Urdal and

Landaas, 1979). None of the patients with these enzyme-immunoglobulin complexes had any underlying pathology in common. Indeed, two individuals with the lactate dehydrogenase-IgA complex and one with the aspartate aminotransferase-IgG complex were apparently healthy. The ALP-IgG complex described here must therefore be seen as only one of a number of such enzyme-immunoglobulin complexes which arise, apparently spontaneously, in the serum of certain individuals with a variety of diseases.

CHAPTER 7.

DISCUSSION

7.1. COMPARISON BETWEEN THE DIFFERENT HIGH MOLECULAR WEIGHT FORMS OF ALKALINE PHOSPHATASE

This thesis is largely concerned with the very high mol wt ALP released into serum in liver disease, but in Chapter 6 another ALP of intermediate mol wt, designated 'slow band ALP', was described in the sera of patients with a variety of diseases. There are some similarities between the two high mol wt forms:

1) Both probably consist of ALP attached in some way to another biochemical substance, as demonstrated by similar immunochemical techniques.
2) Both are inhibited by L-homoarginine but largely unaffected by L-phenylalanine, demonstrating that they belong to the liver/bone class of ALP isoenzymes rather than the intestinal or placental class.
3) Both are slightly more heat-labile overall than the liver isoenzyme.
4) The electrophoretic mobility of both is retarded by neuraminidase treatment, suggesting that they contain sialic acid residues.

However, the differences are more striking than the

similarities:

1) There may be as much as an order of magnitude difference in the mol wts.
2) The substances to which ALP is attached are quite different, namely IgG in the case of slow band ALP and probably membrane fragments in the case of high mol wt ALP (see sections 7.3 - 7.9).
3) The nature of the attachment is different since it is unaffected by detergents in the case of slow band ALP but is ruptured by detergents in the case of high mol wt ALP, suggesting that hydrophobic interaction is involved.
4) The presence of slow band ALP in serum is a rare event which is not specific for any particular disease, although when it does occur it occurs more often than not in intestinal or lung disorders; whereas a raised level of high mol wt ALP in serum is specific for liver disease.
5) Slow band ALP arises from an interaction in vivo between liver or bone ALP and IgG to give soluble complexes, whereas high mol wt ALP probably derives from shedding of membrane fragments from the bile canaliculi into bile in vivo and regurgitation into the circulation (see section 7.9.).

Slow band ALP, in view of its rarity and apparent lack of usefulness in clinical diagnosis, was less intensively investigated than the high mol wt ALP released into the circulation in liver disease. Its nature and possible origins have already been discussed in Chapter 6. The remainder of this chapter will therefore be devoted to a discussion of the nature and possible origins of the high mol wt ALP which appears in the serum of patients with liver disease.

7.2. ELECTROPHORETIC AND CHROMATOGRAPHIC BEHAVIOUR OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE

In order to eliminate any confusion arising from the large number of different analytical methods used in the literature, experiments were carried out to establish the electrophoretic and chromatographic behaviour of high mol wt ALP in various media. These showed that the high mol wt ALP excluded from Sepharose 6B gel is identical to:

1) the ALP which eluted at an ionic strength of 135 mmol/l during salt gradient DEAE-cellulose chromatography
2) the enzyme activity present in Fraction II in the two-step ion exchange assay (section 2.9.)
3) the activity remaining at the origin after electrophoresis in 7% polyacrylamide gel (c.f.

Walker and Pollard, 1971)

4) the enzyme band which migrated a little way into the gel during electrophoresis in 2.5% polyacrylamide gel

5) the enzyme band which migrated immediately behind the liver isoenzyme during electrophoresis in 1% agarose gel (c.f. Demetriou and Beattie, 1971), and between the intestinal isoenzyme and the origin in 2.2% agarose gel (c.f. Ewen, 1974).

6) the isoenzyme which migrated ahead of the liver isoenzyme in the α_1 globulin position during electrophoresis in cellulose acetate (c.f. Fritsche and Adams-Park, 1972)

Starch gel was not studied as an electrophoretic medium because of the slow and tedious nature of the technique and because it has largely been abandoned as the medium of choice in favour of the simpler faster techniques now available. However, it seems likely that high mol wt ALP remains at the origin in starch gel. The ALP band migrating in the βlipoprotein position in starch gel, which may appear both in health and disease, appears to be distinct from the high mol wt ALP which appears in serum only in liver disease. In this connection, Van Husen and Gerlach (1974) found that if the liver isoenzyme and the

βlipoprotein-associated ALP in serum increased in liver disease without an increase in activity at the origin, no concomitant increase was seen in LAP, 5'NT and lipids in serum. On the other hand, if the activity at the origin also increased, these markers of cholestasis also rose. This is further evidence for the fundamental difference between βlipoprotein-associated ALP and high mol wt ALP. This βlipoprotein-associated ALP may constitute the non-specific enzyme staining observed in the βlipoprotein precipitin arcs (section 4.8.), and may represent non-specific sequestering of a wide variety of enzymes by βlipoprotein in serum as suggested by Lawrence and Melnick (1961). This thesis is not concerned with this non-specific βlipoprotein-associated ALP.

By studying the behaviour of high mol wt ALP in these various media, continuity with the literature was made possible. Discussion of the results in detail and comparison with any similar experiments carried out by other authors have already been presented in the relevant chapters. In this chapter, the findings will be combined more generally with other research in various fields to form a unified theory of the nature and behaviour in disease of high mol wt ALP.

7.3. RELATIONSHIP OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE WITH OTHER MEMBRANE MARKER ENZYMES

ALP exists in the liver as a membrane-bound enzyme (Birns et al, 1962) and high mol wt ALP has been shown to share one or more antigenic determinants with liver plasma membrane (Shinkai and Akedo, 1972). Of the various hypotheses which might account for the large size of high mol wt ALP (e.g. association of ALP with either protein, lipid or carbohydrate, or aggregation of ALP itself), the possibility that it might represent circulating fragments of liver cell membrane was therefore taken as the working hypothesis to be tested.

7.3.1. Serum

γGT, LAP, and 5'NT are also membrane-bound enzymes which are released into serum in liver disease. Investigation showed that they too had high mol wt components (section 4.6.), and this has been confirmed by Shinkai and Akedo (1972), de Broe et al (1975) and Huseby (1978). Moreover, the activities of the high mol wt components of all four membrane marker enzymes appeared to vary roughly in parallel in serum. No high mol wt component was found for the cytoplasmic enzyme, lactate dehydrogenase, in a serum which contained high mol wt ALP, γGT, LAP and 5'NT. It seems likely therefore that only enzymes which are of

membrane origin give rise to high mol wt components in serum. All four high mol wt enzymes migrated with identical mobilities during electrophoresis and eluted in the same void volume peak during gel filtration chromatography. Supporting circumstantial evidence from previous authors, each studying one enzyme only, suggests that γGT, LAP and 5'NT all have bands which remain at the origin on starch gel or polyacrylamide gel electrophoresis in serum from patients with liver disease but not from healthy individuals (Orlewski and Szczeklik, 1967; Azzopardi and Jayle, 1973; Kowlessar and Haeffner, 1961). In addition, de Broe et al (1975) showed that the high mol wt ALP band on agar gel electrophoresis also stained for γGT activity.

At first sight, the different elution positions of high mol wt ALP, γGT and LAP on ion-exchange chromatography would seem to contradict this hypothesis. However, all the peaks overlapped to some extent and it must be remembered that membrane fragments are not necessarily homogeneous. If one therefore postulates a family of membrane fragments of large molecular size, to each of which are attached the enzymes in different proportions vis à vis each other, then this would give the observed pattern of sequential but overlapping peaks. This is in agreement with recent theories of membrane structure which

no longer regard the membrane as a uniform entity consisting of a phospholipid bilayer interspersed with an even distribution of protein, but as a mosaic of functionally and morphologically distinct regions of different protein composition. Such mosaicism of enzyme localisation has been demonstrated for a number of membranes including plasma membrane, endoplasmic reticulum, chloroplasts and bacterial cell membranes (Siekevitz, 1972). Moreover, the enzymes in these membranes may be induced or repressed independently of each other. Therefore, although high mol wt ALP, γGT, LAP and 5'NT appeared to be roughly correlated, such correlation is not a necessary corollary of the membrane fragment hypothesis.

7.3.2. Bile

In normal human hepatic bile, only plasma membrane enzymes are found in relative abundance: there is a relative lack of intracellular enzymes (Holdsworth and Coleman, 1975). The investigations presented in this thesis demonstrate that, like serum in patients with liver disease, normal bile contains high mol wt ALP, γGT and LAP (section 4.6.) in addition to low concentrations of the low mol wt isoenzymes. In electrophoretic and chromatographic properties these high mol wt biliary enzymes closely resemble the high mol wt serum enzymes.

7.4. EFFECT OF DISSOCIATING AGENTS

7.4.1. Butanol

Butan-1-ol has frequently been employed to solubilise membrane-bound proteins by removal of the lipid phase (Morton, 1954). Conflicting reports of its effects on high mol wt ALP have been reported in the literature (see section 4.7.1.). These discrepancies probably arise from the varying severity of the effect of this powerful organic solvent on enzyme structure under different extraction conditions. In the present study, high mol wt ALP, γGT and LAP from both serum and bile were all apparently destroyed by butanol without conversion to a lower mol wt form, under conditions which did not denature the low mol wt isoenzymes. This suggests that these high mol wt enzymes are associated with a lipid moiety and is consistent with the membrane fragment hypothesis. It is probable that the high mol wt enzymes were extracted into the butanol layer, together with the membrane fragments to which they were attached, and there denatured.

7.4.2. Detergents

Detergents have been employed as rather gentler agents in the solubilisation of membrane proteins. These are amphiphilic substances which solubilise membrane-bound proteins by the combined interaction of their nonpolar

'tails' with the nonpolar portion of the protein (which is hydrophobically linked to the lipid-rich membrane), and their polar 'heads' with the water molecules. By similar mechanisms, detergents disrupt the structure of the membrane lipid bilayer itself. This interaction makes solubilisation energetically favourable, resulting in an increase in entropy (Helenius and Simons, 1975). Since ALP is an integral protein in the hepatocyte membrane i.e. tightly bound to the membrane, this is the only means of achieving solubilisation.

When the more powerful anionic detergents such as SDS are used, they may cause, in addition, dissociation of polypeptide chains and conformational changes in tertiary structure since protein-protein interaction is also affected, leading to loss of enzyme activity. In the present study, this explains the loss of γGT and LAP activity in the presence of SDS. It is thought that the sialic acid residues of ALP in some way protect it against such denaturation (Trépanier et al, 1976).

Triton X-100 converted the high mol wt forms of ALP, γGT and LAP from serum and bile to lower mol wt forms (section 4.7.2.). However, SDS, the more powerful detergent, resulted in conversion to even lower mol wt forms (section 4.7.3.). Similarly, in the solubilisation of

rat liver nucleotide pyrophosphatase, Bachorik and Dietrich (1972) noted that SDS reduced the size of membrane fragments obtained by Triton X-100.

Following solubilisation by detergents, membrane proteins exist in the form of protein-detergent complexes whose mol wts are dependent on the degree of hydration and the number of detergent molecules associated with each protein molecule. This may explain the mol wt of 410 000 for the SDS-solubilised high mol wt ALP compared with 220 000 for the liver isoenzyme. There may be a variety of sizes of complexes in equilibrium, the relative proportions of which depend on the conditions. This may partly explain the range of mol wts observed for the Triton X-100-solubilised high mol wt ALP, although partial re-association of the membrane fragments, a frequent occurrence following reduction in Triton X-100 concentration, may also have been responsible.

It was not considered worth while to attempt complete removal of the detergents following solubilisation in order to measure the true mol wt of the solubilised protein and compare it with the liver isoenzyme to check whether the two might be identical. This was because complete removal of detergents is very difficult since they are largely present in micellar form and therefore dialyse

very poorly. Even when it can be brought about it usually leads to protein aggregation, often to the extent of formation of amorphous precipitates, probably as a result of hydrophobic interaction (Helenius and Simons, 1975). Nevertheless, the evidence is compatible with the hypothesis that the high mol wt enzymes consist of the normal low mol wt liver isoenzymes attached to membrane fragments which can be disrupted by detergents.

7.4.3. Papain

Relevant to the detergent studies presented here is a study on the effects of the proteolytic enzyme, papain, on the LAP activity associated with rat liver plasma membrane (Emmelot et al, 1968). It was noted that papain released LAP almost completely from most membranes and that this coincided with the release of 6nm globular knobs seen under the electron microscope on the surface of the membrane lining the bile canaliculus. It was concluded that LAP was located on these knobs which represented a structurally and functionally specialised repeating unit on the membrane.

Recently, Huseby (1978), using a detergent combination of deoxycholate and Lubrol W, converted high mol wt γGT from serum and bile to a lower mol wt form. Treatment with papain had a similar effect. He explained these

findings by postulating hydrophobic domains on the low mol wt form of the enzyme which might complex with lipids or proteins in serum *in vivo* following release into the circulation in liver disease. However, his observations could equally well be explained by the release into the circulation of membrane fragments consisting of lipid and structural protein to which γGT is attached, as suggested by the membrane fragment hypothesis.

In the series of investigations presented here, the effects of bile acids (which are detergents) and papain on the high mol wt enzymes in serum and bile have not yet been studied. This would be a worthwhile direction for further research to take since the ability of papain to disrupt all four high mol wt enzymes would suggest the presence not only of lipids (as shown by the butanol and detergent experiments) but also of structural protein in the complexes, thereby providing additional evidence for the membrane fragment theory.

7.5. HEAT INACTIVATION

There are a number of possible explanations for the bi-exponential heat inactivation curves of serum and biliary high mol wt ALP (section 4.5.) based on the hypothesis that they consist of ALP attached to membrane fragments:

1) Two different forms of ALP with different heat

stability may be attached to a single membrane fragment.

2) A single form of ALP may be attached to two different membrane fragments whose differing compositions confer different degrees of heat stability on the attached ALP.

3) The membrane fragments may be relatively homogeneous but the ALP molecule may be attached to two different sites on the membrane which confer different degrees of heat stability.

No hard evidence is available to distinguish between these possibilities although the otherwise homogeneous properties of high mol wt ALP make (1) unlikely. They are all consistent with the membrane fragment hypothesis.

7.6. KINETICS

The observed fact that high mol wt ALP from both serum and bile obeys the same general kinetics as the liver isoenzyme and the low mol wt biliary isoenzyme, with regard to optimum conditions of assay, mechanism of inhibition by L-homoarginine and activation by magnesium (Chapter 3), supports the hypothesis that these isoenzymes may all derive from similar sources and be genetically coded at the same locus. The observation of differences in K_ms, K_is and K_As between serum high mol wt ALP and

biliary high mol wt ALP, biliary low mol wt ALP and the liver isoenzyme does not contradict this theory, since a general characteristic of membrane enzymes is a difference in such kinetic properties between the membrane-bound and solubilised forms. This phenomenon is known as "allotopy" and arises from conformational changes between the two states (Farias et al., 1975). In other words, the presence of the membrane structure will affect the catalytic parameters of the enzyme, although the same fundamental mechanism of catalysis will be retained.

7.7. CHANGES IN LIVER CELL HISTOLOGY IN CHOLESTASIS

In order to develop the membrane fragment theory further and provide an explanation for the release of high mol wt enzymes in liver disease, it is necessary to take into account the morphological and enzymic changes in liver cell histology following either experimental bile duct ligation in laboratory animals or biliary obstruction in humans.

In normal rat and human livers ALP is principally located on the sinusoidal membrane with only faint activity associated with the bile canalicular membrane. (Birns et al, 1962; Hägerstrand 1975). LAP is also associated with the liver plasma membrane, particularly that lining the bile canaliculi (Emmelot et al, 1968).

5'NT is localised both on the sinusoidal and the bile canalicular membrane and also frequently along the canalicular-sinusoidal connections (Birns et al, 1962).

Following bile duct ligation in rats, the bile canaliculi are first distended and become tortuous owing to the pressure of bile and then an increasing number of canalicular-sinusoidal connections open up (Birns et al, 1962). This is accompanied by an increasing amount of 5'NT lining these connections and the canaliculi also begin staining for ALP, this staining reaching its greatest intensity 24 hours after bile duct ligation. The sinusoidal membrane also stains for ALP and this staining is always more intense than that of the bile canaliculi. On the other hand, rats who have been exposed to carbon tetrachloride, which causes liver cell necrosis rather than extrahepatic obstruction, show only sinusoidal ALP staining and no ALP staining of the bile canaliculi (Wachstein and Meisel, 1958).

In liver disease in humans, hepatitis and related diseases causing liver cell necrosis result in increases in the sinusoidal membrane ALP but no detectable activity associated with the bile canaliculi (Hägerstrand, 1975). However, in extrahepatic obstruction and metastatic liver disease and, to a lesser extent, in cirrhosis and chronic

active hepatitis, dilatation and ALP staining of the bile canaliculi is observed in addition to the more intense ALP staining of the sinusoidal membrane and, as in the rat, increasing numbers of canalicular-sinusoidal connections appear (Wachstein and Meisel, 1958; Hägerstrand, 1975). The appearance of ALP staining of the bile canaliculi therefore occurs under the same pathological conditions in which high mol wt ALP appears in largest amounts in the circulation (Chapter 5).

Looking now at the properties of ALP following bile duct ligation in rats, Kaplan and Righetti (1969) claimed that no new isoenzymes appeared in serum following ligation. Nevertheless their photographs show a steady increase in previously non-existent activity at the origin (corresponding to high mol wt ALP) on polyacrylamide gel electrophoresis over the 24 hour period following ligation i.e. at the same time as the bile canaliculi stain most intensely for ALP (Birns et al, 1962). Moss et al (1974) in similar experiments showed an increase in the heat stability of whole liver ALP within 24 hours following bile duct ligation and this was even more marked after 10 days.

Taking the evidence from humans and rats together, one may therefore postulate that high mol wt ALP derives

principally or wholly from the membranes lining the bile canaliculi and that the liver isoenzyme derives principally or wholly from the sinusoidal membrane of the hepatocyte. The observed increase in heat stability following bile duct ligation could then be explained by the greater increase in sinusoidal ALP (liver isoenzyme) than in canalicular ALP (high mol wt ALP) which is less heat stable (section 4.5.).

7.8. RELATIONSHIP BETWEEN HIGH MOLECULAR WEIGHT ENZYMES AND LIPOPROTEIN X

LPX is an abnormal lipoprotein which appears in the serum of patients with obstructive jaundice (Seidel et al, 1969) and, much more rarely, in patients with a deficiency of lecithin-cholesterol acyl transferase (LCAT) which governs the esterification of cholesterol from phospholipid. In cholestasis, the appearance of LPX in serum tends to be inversely correlated with LCAT activity i.e. when LCAT activity is low (as often happens in biliary obstruction) LPX can be detected, but when, as sometimes happens, LCAT activity is normal or high LPX is not detected (McIntyre, 1978). This close relationship ties in with the composition of LPX which is rich in phospholipid and unesterified cholesterol, with esterified cholesterol and triglyceride virtually absent (Seidel et

al, 1969). About 2% of the molecule is protein, mainly apo-C and albumin. LPX has been shown to exist in heterogeneous forms of varying molecular size, one or more of which are, like the high mol wt enzymes, excluded by Sepharose 4B (Brocklehurst et al., 1976; Hauser et al, 1977). Electron microscopy of purified LPX revealed various sizes of hollow vesicles surrounded by a membran--ous bilayer, with a distinctive tendency to be grouped in stacks (Hauser et al, 1977). Following bile duct ligation in rats, LPX appeared in the serum after a few hours, reached a peak at 40 hours and thereafter showed a progressive fall to become undetectable after approximately one week (Seidel et al, 1976). This time course parallels that of total ALP following bile duct ligation.

There is some evidence that high mol wt ALP may have a chemical composition similar to that of LPX. Price et al (1972) found that butanol extracts of high mol wt ALP from bile contained principally phospholipid (which is the principal component of membranes also). In chloroform: methanol extracts of high mol wt ALP from serum, Shinkai and Akedo (1972) found a mixture of phospholipid, cholesterol, triglyceride and free fatty acid, although the relative proportions were not measured. Electron microscopy of concentrated pellets of high mol wt ALP

from serum showed vesicles similar to those of LPX which stained to varying extents for ALP (Brocklehurst et al, 1976) and 5'NT (de Broe et al, 1975). Since such vesicles may occur in any particle with approximately the right lipid composition, this is further evidence that the chemical composition of LPX and the high mol wt enzymes may be similar.

The experiments described in section 4.8. indicate that the high mol wt enzymes share antigenic determinants with LPX and also migrate cathodally with LPX during electrophoresis in agar gel. The evidence for the immunochemical and electrophoretic identity of high mol wt ALP, γGT, LAP and 5'NT with LPX is, however, counterbalanced by the lack of detectable LPX in bile which nevertheless contains these high mol wt enzymes. It is also counterbalanced by the evidence presented in section 4.8.3. that although sera containing detectable LPX invariably had raised levels of high mol wt ALP, many sera possessing high activities of high mol wt ALP did not contain detectable LPX. This was also found by de Broe et al (1975). Such observations need not necessarily conflict with the theory that the high mol wt enzymes and LPX consist of membrane fragments of similar or identical composition. There are two possibilities consistent with this hypothesis.

Firstly, the membrane fragments to which the enzymes are attached may always have an identical composition which can be immunologically and electrophoretically identified as that of LPX. However, the number of enzyme molecules associated with a given LPX particle might vary (again the concept of membrane mosaicism) so that large amounts of high mol wt enzyme activity might be associated with low amounts of LPX, undetectable by the methods used. This does not satisfactorily explain why, when LPX can be detected, the enzyme activities associated with it appear to vary in parallel with each other and with LPX.

The second possible explanation is that the high mol wt enzymes, although always associated with membrane fragments of some kind (as may LPX), may not necessarily be invariably associated with membrane fragments which behave immunochemically and electrophoretically as LPX. Slight differences in membrane composition might alter the characteristic electrophoretic mobility of LPX in agar gel or make the membrane undetectable by LPX antiserum, even at high concentration. When the membrane is detectable as LPX, though, the enzyme activities associated with it would be correlated with it, as was observed.

Such a difference in membrane composition may be brought about by contact with high circulating concentrations

of bile salts which are known to alter the characteristic electrophoretic migration of LPX from cathodal to anodal (Manzato et al, 1976). This would explain why LPX is never detected in bile even when large amounts of high mol wt enzymes are present. There is some evidence to show that lipoprotein lipase may have a similar effect (Sauar et al, 1978).

Relationship between LPX and biliary lipoprotein

Detailed studies of the lipoprotein composition of hepatic bile have shown that its lipid composition is almost identical to that of LPX but that its apoprotein is albumin rather than apo-C and the protein:lipid ratio is different (Manzato et al, 1976). It migrated towards the anode rather than the cathode during electrophoresis in agar gel. Under the electron microscope it formed lamellar structures rather than the stacked vesicles characteristic of LPX. However, it could be converted *in vitro*, by the addition of serum to the native bile, to a lipoprotein which was identical to LPX in its electrophoretic, immunological and morphological properties. At least part of this transformation involved the association of Apo-C from serum with the biliary lipoprotein. This conversion was critically dependent on the bile salt: albumin concentration ratio and did not take place if

this ratio increased or decreased beyond certain limits. Conversely, LPX could be converted to a lipoprotein resembling the biliary lipoprotein in every way by the addition of bile salts to a LPX-positive serum *in vitro*. In dogs, when the common bile duct was experimentally connected to the vena cava *in vivo*, LPX appeared in the plasma after 3 hours but after 8 hours rapidly declined, presumably owing to altered serum factors e.g. bile acid concentration. This evidence strongly suggests that the biliary lipoprotein is a precursor of LPX which refluxes into the plasma pool during cholestasis. Whether or not LPX is formed depends critically on the serum bile salt:albumin ratio and possibly other factors also.

Two slightly different forms of the high mol wt enzymes must therefore be postulated, one immunochemically and electrophoretically identical to the biliary lipoprotein and the other to LPX. Bile would contain only the biliary lipoprotein-enzyme complex, whereas serum would contain either the biliary lipoprotein-enzyme complex or the LPX-enzyme complex or even both in equilibrium depending on various serum factors. This would be consistent with the biphasic heat inactivation curve for high mol wt ALP since this was purified from more than one serum (section 4.5.), and also with the observation that two kinds

of high mol wt ALP, LAP and 5'NT were found on sucrose density gradient ultracentrifugation, one in the 6.3 S region and the other in the 17.5 S region (Shinkai and Akedo, 1972). The attachment of ALP to two membrane fragments of slightly differing composition would not alter the mechanism of catalysis, activation or inhibition but might alter some of the parameters, and this was observed for the K_ms in particular (section 3.2.4.).

The observation that purified high mol wt ALP from serum and from bile did not migrate on agar gel electrophoresis, but precipitated around the sample well during LPX immunoelectrophoresis (section 4.8.2.) is consistent with the observation by Manzato et al (1976) that purified biliary lipoprotein also failed to migrate, requiring the presence of bile salts to exhibit its characteristic anodal migration. High mol wt enzymes from both serum and bile were, however, both specifically precipitated by incubation with LPX antiserum (section 4.8.2.) suggesting that, although the biliary-enzyme complex did not have detectable cathodally-migrating LPX, it did share antigenic determinants with the LPX-enzyme complex. What these determinants might be is uncertain since it seems that the biliary lipoprotein does not contain Apo-C (Manzato et al, 1976). Studies similar to those carried out by Manzato et

al (1976) on the effects on the high mol wt enzymes and their association with LPX of a) adding albumin, Apo-C or whole serum to bile and b) adding bile salts to serum might help to elucidate this point and to confirm or refute the theory outlined of two types of high mol wt ALP.

The concept of two main types of membrane fragment in serum need not conflict with the previous postulate that the various membrane-marker enzymes are associated with membrane fragments in varying proportions depending on the exact position in the bile canalicular membrane mosaic from which they are derived. These would form sub-classes of membrane fragments. It must be remembered that LPX itself as an immunochemically distinct species is heterogeneous (Hauser et al, 1977) and so may derive from a number of localities in the membrane mosaic.

An alternative theory

One alternative theory to the membrane fragment hypothesis, but incorporating many of its features, should be mentioned. This is that LPX may not consist of fragments of membrane (containing membrane marker enzymes) to which Apo-C has been added in the circulation, but simply of a lipoprotein of altered composition arising in a way similar to that of βlipoprotein. The low mol wt enzymes may then attach themselves to LPX following their release

into the circulation, to form high mol wt enzymes. This attachment would presumably be a hydrophobic one since it could be disrupted by organic solvents and detergents. A similar attachment might occur between the low mol wt enzymes and biliary lipoprotein. It is difficult to distinguish between this possibility and the membrane fragment hypothesis on the evidence so far available. However, the theory is rendered less likely by the experiments of Manzato et al (1976) which demonstrated that the precursor of LPX almost certainly comes from bile and not from altered synthesis of very low density lipoprotein (the precursor of low density lipoprotein) in the hepatocyte followed by direct release into the circulation. Furthermore, only membrane-marker enzymes seem to be attached to LPX and, as shown by Shinkai and Akedo (1972), high mol wt ALP shares antigenic determinants with liver plasma membrane.

7.9. FORMATION OF HIGH MOLECULAR WEIGHT ENZYMES IN CHOLESTASIS

In health, all membrane constituents are involved in dynamic turnover in the steady state (Siekevitz, 1972). On a larger scale, many intact viable cells appear to shed membrane fragments *in vivo*, the composition of the fragments resembling that of the intact membrane (de Broe et

al, 1977). This may represent a normal physiological turnover of the membranes, although the rate of shedding may be increased if there is local degeneration of the cells or a modified cellular environment. It is probable that such shedding of the bile canalicular membrane into bile occurs in the healthy individual to form the high mol wt enzymes which are present in normal hepatic bile.

The pathological process resulting in the appearance of high mol wt ALP and LPX in the circulation may be as follows. During extrahepatic or intrahepatic cholestasis, ALP synthesis is induced both on the sinusoidal and on the bile canalicular membrane. In some way, the ALP on the sinusoidal membrane is solubilised, possibly by the detergent action of the increased local concentration of unexcreted bile salts, resulting in an increase in the normal liver isoenzyme in serum. At the same time there may be increased shedding of fragments from the bile canalicular membrane (and possibly also from the sinusoidal membrane) owing to the modified environment and possible cellular degeneration. Some of this increased shedding may be due to altered membrane composition as a result of reduced LCAT activity and some may be brought about by a less complete action of bile salts. Some of the fragments may pass into bile as high mol wt enzyme

complexes; these would be equivalent to biliary lipoprotein. They may then be regurgitated along with bile into serum via the increased number of bile canalicular-sinusoidal connections which open up in cholestasis. The biliary lipoprotein membrane fragments may be converted to fragments immunochemically and electrophoretically identical with LPX, depending on various serum factors including bile salt and albumin concentration, and possibly lipoprotein lipase and LCAT activities. It is possible that some fragments are also shed directly into serum from the sinusoidal membrane.

It has been observed that administration of lithocholate to normal rabbits resulted in the appearance of LPX in their serum (James et al, 1974). If LPX is indeed equivalent to membrane fragments, this observation is consistent with the argument that increased shedding of bile canalicular and sinusoidal membrane fragments may occur in the presence of increased concentrations of bile salts in cholestasis.

Metastatic liver disease

The explanation for the particularly high levels of high mol wt ALP in serum in metastatic liver disease in the absence of jaundice and usually in the absence of detectable LPX (Chapter 5) may be as follows. Firstly, local deposits

of tumour stimulate preferential induction of bile canalicular ALP (Hägerstrand, 1975) and this may shed at an increased rate into bile. Reflux of bile occurs into serum owing to local intrahepatic obstruction by the tumour, carrying with it biliary lipoprotein-associated high mol wt ALP. Bilirubin will of course also be regurgitated but can be re-excreted by another unaffected portion of the liver, so that jaundice does not occur. High mol wt ALP, on the other hand, cannot be excreted by the liver and remains in the circulation. Biliary lipoprotein-associated high mol wt ALP rarely seems to be converted to LPX-associated high mol wt ALP under these circumstances, possibly because of high circulating bile salt concentrations (which were not measured in the study) or other altered serum factors such as high lipoprotein lipase or LCAT activities, both of which also result in the disappearance of cathodally-migrating LPX (Sauar et al, 1978; McIntyre, 1978).

Viral hepatitis

In viral hepatitis, where slight increases in high mol wt ALP were shown to occur roughly in parallel with total γGT and LAP (section 5.8.), these may result from an increased rate of shedding of membrane fragments into the sinusoid, since viral infection attacks liver cell

membranes. In addition, cholestasis caused by intrahepatic obstruction of the bile canaliculi by swollen parenchymal cells may cause increases in high mol wt ALP by the mechanisms outlined in the preceding paragraph.

In all these diseases, the actual amount of high mol wt ALP in the circulation may depend on:

1) the degree of induction of the bile canalicular membrane ALP
2) the rate of shedding of bile canalicular membrane fragments into bile and serum which itself may depend on local factors such as bile salt concentration and cellular degeneration
3) the degree of biliary obstruction and therefore of bile reflux
4) the number of canalicular-sinusoidal connections
5) the rate of removal of high mol wt ALP from the circulation possibly partly by inactivation and partly by uptake by the reticulo-endothelial system.

Whether or not the high mol wt ALP is associated with cathodally-migrating LPX may depend on the circulating albumin and bile salt concentration, lipoprotein lipase and LCAT activity and possibly other, as yet unknown, serum factors.

7.10. CLINICAL EVALUATION OF HIGH MOLECULAR WEIGHT ALKALINE PHOSPHATASE

Turning from general theoretical considerations to the practical clinical evaluation of high mol wt ALP as outlined in Chapter 5, this was necessarily limited by the problems which affect all such studies. One such problem is variation in the severity of disease from one patient to the next within any given diagnostic category, giving rise to a range of possible pathological processes e.g. cholestasis, cell necrosis, loss of biosynthetic capacity. Another problem was that only one sample was taken for discriminant function analysis, and the timing of the samples varied from patient to patient in relation to the time course of their disease. Both these factors tended to widen the range of biochemical results within a given disease and to blur the distinction between diseases. But these problems are also likely to be encountered in routine practice; therefore, the conclusions derived from this clinical evaluation are likely to be sufficiently robust to apply to routine diagnostic practice.

The other limitation of the study relates to the criterion that only patients with raised ALP levels were included, thereby biassing the results. This was done because at this stage only a pilot study was contemplated

to assess whether high mol wt ALP offered more than total ALP in the diagnosis of liver disease, and some form of pre-selection seemed desirable to focus attention on those patients who were definitely ill. More clinical studies on a prospective basis on larger numbers of patients, including those with normal total ALP activities, would be required to assess fully the role of high mol wt ALP in diagnosis. Further serial studies might also elucidate its role in management, for example as a marker for liver secondaries.

Nevertheless, the clinical evaluation provided a useful guide to the value of high mol wt ALP in routine diagnosis and highlights the areas which may repay further research. On the one hand, the study has shown that, although high mol wt ALP may contribute to the decision as to whether a raised ALP activity is of liver or bone origin, there are other simpler tests, which do this equally effectively. Nor is high mol wt ALP more effective in the diagnosis of viral hepatitis than the tests currently in common use. On the other hand, high mol wt ALP was a sensitive test for obstructive liver disease, particularly local intrahepatic obstruction caused by liver metastases. It seems possible, therefore, that high mol wt ALP may be a sensitive test for detecting early liver metastases

where there is a known primary carcinoma. This would be a rewarding area for further research.

APPENDIX 1

Abbreviations made in the text

In general, the trivial names of enzymes recommended by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (1972), have been employed, with the exception of aminopeptidase which has been termed leucine aminopeptidase in order to maintain continuity with the literature. A list of the abbreviations used in the text together with the systematic name (for enzymes) and Enzyme Commission code number follows:

Abbreviation	Trivial name	Systematic name	Code number
ALP	Alkaline phosphatase	Orthophosphoric-monoester phosphohydrolase (alkaline optimum)	3.1.3.1.
γGT	γ-Glutamyltransferase	(γ-Glutamyl)-peptide: amino acid -glutamyltransferase	2.3.2.2.
LAP	Leucine aminopeptidase	α-Aminoacyl-peptide hydrolase	3.4.11.1/2
LCAT	Lecithin-cholesterol acyltransferase	Lecithin-cholesterol acyltransferase	2.3.1.43
LPX	Lipoprotein X	-	-
5'NT	5'Nucleotidase	5'-Ribonucleotide phosphohydrolase	3.1.3.5.

In addition a few further abbreviations have been used in some tables and figures:

Abbreviation	Full name
Alb	Albumin
ALT	Alanine aminotransferase
Bili	Bilirubin
HMW or High MW alk phos	High molecular weight alkaline phosphatase

In Chapter 5 the diagnostic categories were abbreviated in figures and tables as follows:

Abbreviation	Diagnostic category
Normals	Normal controls
Bone disease	Osteomalacia and metastatic bone disease
Acute hep.	Serum hepatitis and infectious hepatitis
C.A.H.	Chronic active hepatitis
P.B.C.	Primary biliary cirrhosis
Alc. cirr.	Alcoholic cirrhosis
Ca. pancreas	Carcinoma of the head of the pancreas
Chol. ca.	Cholangiocarcinoma
Gallstones	Gallstone obstruction/cholecystitis
Liver 2^{o}s	Metastatic liver disease

APPENDIX 2

Publications

One paper covering an aspect of the work presented in this thesis has been published:

Crofton P M, Smith A F. The properties and clinical significance of some electrophoretically slow forms of alkaline phosphatase. Clinica Chimica Acta 1978; 83: 235-247.

A further two papers have been accepted for publication in Clinica Chimica Acta:

Crofton P M, Smith A F. An ion-exchange assay for high molecular weight alkaline phosphatase.

Crofton P M, Smith A F. High molecular weight alkaline phosphatase: a clinical study.

APPENDIX 3

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The work for this thesis was carried out while I was employed as a Lecturer in the University Department of Clinical Chemistry, Royal Infirmary, Edinburgh. All the practical work has been performed personally with the exception of the routine assays of alanine aminotransferase, albumin, alkaline phosphatase and bilirubin presented in Chapter 5, which were performed by the technical staff of the Department of Clinical Chemistry, Royal Infirmary, Edinburgh.

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